Chapter - III

Materials and methods
Coronary Artery Disease (CAD): Both newly or previously diagnosed types of CAD are recognized based on clinical information and supported by at least one objective evidence. This includes previous myocardial infarction and revealed by a resting ECG. Myocardial ischemia is revealed by abnormal stress tests by means of exercise ECG, echocardiography, magnetic resonance imaging, coronary angiogram with more than 50% stenosis of lumen diameters in any major coronary artery or a previously documented cardiovascular event (hospitalization for acute coronary syndrome, previous myocardial infarction, percutaneous coronary intervention (PCI) or coronary artery by-pass grafting (CABG).1,2

Acute myocardial infarction (MI): All the studies are followed the definition according to the joint recommendations by ESC and ACC, with markers of myocardial ischemia exceeding the upper reference limit on two occasions (troponin T > 0.05 g/l or CK-MB > 10 ug/l) in the presence of typical symptoms (chest pain > 15 min; pulmonary oedema in the absence of valvular heart disease, cardiogenic shock, ventricular tachycardia or ventricular fibrillation) or new Q-waves in at least two of the twelve standard ECG leads or ECG indicating acute ischemia (ST segment elevation, ST-depression or T-wave inversion).

Previously known diabetes mellitus: It has been ascertained if its diagnosis of diabetes has been established before patient enrolment, and this study is classified into four main etiological types: diabetes type 1, type 2, gestational type, or other specific types.3 Glucometabolic status in patients without previously known diabetes history has been classified into different categories according to the diagnostic criteria issued by the World Health Organization3 and the American Diabetes Association.4,5 WHO recommendations, a standardised oral glucose tolerance test (OGTT; 75 gm glucose in 200-250 ml water) together with these considered the "golden" standard for the assessment of glucose metabolism in all studies, unless there is presence of overt hyperglycaemia.3

Dyslipidaemia: It has been applied as a term in the presence of HDL-cholesterol < 34.7 mg/dl in men and < 38.61 in women and/ or triglycerides > 150 mg/dl.3
Hyperlipidaemia: It has been recognized, in case of lipid profile exceeding the reference limits recommended in the European Guidelines for Cardiovascular Disease Prevention (total cholesterol > 193 mg/dl HDL-cholesterol < 38.61 mg/dl in men, or < 42.5 mg/dl in women, triglycerides > 177 mg/dl) or ongoing lipid-lowering treatment.6

Obesity: Body Mass Index (BMI) is the parameter used to determine obesity. Height is measured to the nearest whole centimeters (cm) and weight to the nearest whole kilogram (kg). BMI is calculated as weight (in kg) divided by height (in meters) square. Obesity is recognized if the body mass index (weight/height$^2$), BMI exceeded >30.

Blood pressure: It is measured on the right arm, after 10 minutes rest, in the recumbent position and, after another 2 minutes, in the sitting position. Mercury manometers (Kifa Ercameter, wall-model) are used. Systolic and diastolic blood pressures are read to the nearest 5 mm Hg. The diastolic blood pressure is recorded, as usual, at the disappearance of the Korotkoff sounds.

Definition of hypertension: It is based on questionnaire information and blood pressure measurements; the criteria adopted are self-reported use of antihypertensive treatment or systolic blood pressure >150 mm Hg and diastolic blood pressure >100 mm Hg.

ECG
ECG is performed for all patients on admission (inclusion ECG), at randomisation, before and after revascularisation. Rhythm, presence and location of pathologic Q waves, ST elevation, ST depression and T wave inversion are recorded. ST depression is considered present if it is horizontal or down sloping and at least 0.05 mV below the isoelectric (P-R segment) level at 60 milliseconds after the J point.
**Troponin T**

Third generation TnT assay (Elecsys, Roche Diagnostics) is done. This assay uses a human recombinant cardiac TnT for calibration, which has substantially improved the precision and sensitivity of the test. The lower limit of detection is 0.01 μg/L, and the upper reference level of healthy individuals is at the same level, according to the manufacturer. At this level, however, the CV is unacceptably high. The functional sensitivity, defined as the level with a CV < 20%, of the assay is 0.03 μg/L, which therefore is used as the cut-off level.

**Statistical methods**

In all experimental studies, Student’s t-test for dependent or independent parameters is done to assess the statistical significance of different data sets. Or, in certain cells, Mann-Whitney U-test is applied. Pearson χ² analysis is used to test the significance of the overall degree of association. Stepwise Forward Logistic Regression Analysis is used to adjust the established risk indicators regarding mortality.

The statistical evaluation is performed on an intention-to-treat basis. Differences between categorical variables are analysed for significance by Pearson (linear-by-linear association or Fisher’s exact test, whichever was applicable) Chi-square test with results presented as risk ratios with 95% confidence intervals. For continuous variables, the analysis of variance (ANOVA) model is carried out to assess significance of differences. Multivariable logistic regression analysis are used to identify independent risk indicators for future cardiac events. For all tests, p<0.05 is considered significant.

The data processing and statistical analyses are performed using the Statistica™ (StatSoft) or SPSS 10.0 or 10.1 statistical programme.

**Pearsonian Correlation Co-efficient**

To find out the interrelationship between different biochemical parameters, CAD risk factor conditions along with age and sex, Pearsonian Correlation Coefficient technique is used. All characters under consideration are studied through the correlation matrix, where each of diagonal elements are simple correlation coefficient between that row and column characters. Significance of correlation is tested at 5% and 1% level. Simple
correlation coefficients are calculated between H while all other parameters for each site and significance of correlation is studied by t-test.

**Multiple Regression Technique**

In biochemical problems if there are intercorrelations between the principal component and explanatory variables, it becomes difficult or impossible to disentangle their separate individual effects on the dependent variable, and regression coefficients tend to have large standard errors. In such a situation, it is a convention to perform a PC analysis on the explanatory variables and regress the dependent variable on some or all of the principal components, since the latter are uncorrelated.

**Generalized Multiple Regression Technique**

In the present study a generalized multiple regression analysis is also performed. Regression equation of the following general form are obtained at each risk factor criteria

\[ Y = a + b_1x_1 + b_2x_2 + \ldots + b_ix_i + \ldots + b_nx_n \]

where,

- \( a \) = Intercept value, a constant.
- \( b_i \) = Respective partial regression coefficient.
- \( x_i \) = The i-th component variable.
- i = Variable number.
- n= No. of variables.
- Y = The estimated dependent variables.

**ANOVA two-way technique**

Two-way analysis of variance technique is used and tested for significance following F tests. Duncan’s test at 5% level of significance is followed in order to compare the groups.

**Sample collection**

Patients attended at the Calcutta Heart Clinic and Hospital are invited to take part in this study after duly informed about the study. They agreed to participate and give their written consent. The mean age is 51 years (range 20-80). Each subject is interviewed by
a physician on a previously completed questionnaire and is examined for measurement of height, weight, and blood pressure. A venous blood sample is drawn for measurements from the subjects having fasted for at least 12 hours. Blood samples are collected from myocardial infarcted patients within 1 hour of patient’s complaint of chest pain.

Sample preparation

Whole blood: Venous blood is drawn into ethylenediaminetetracetic acid (EDTA) tubes and in plain tubes. Thereafter centrifuged at 3000 rpm for 10 min at 4°C. Haemolytic, icteric or turbid samples are excluded. Plasma and serum are separated for the assay. The buffy coat is removed from the EDTA blood and the remaining erythrocytes are removed from the bottom. The cells are washed three times in cold saline (9.0 g/l NaCl) and then haemolysed by the addition of an equal volume of ice-cold demineralized ultrapure water (MilliQ plus reagent grade Millipore) to yield a haemolysate. The aliquots are prepared for assay.

Separation of serum: The blood is allowed to clot in the centrifuge tube for 30 to 45 minutes at room temperature and then the clot is gently removed into a disposable polypropylene tube. The tube containing the serum is then centrifuged for 5 minutes at 3000 rpm. The supernatant serum is then transferred into a dry labeled and stoppered micro centrifuge tube and stored at 2°C to 8°C for estimation of the following parameters.

Parameters for biochemical evaluation

The collected samples of blood and serum from each of the groups of experiment are utilized for estimation of the following parameters:

1. Blood glucose
2. Hemoglobin (Hb%)
3. Total cholesterol
4. Triglyceride (Tg)
5. High density lipoprotein cholesterol (HDL-C)
6. Low density lipoprotein cholesterol (LDL-C)
7. Superoxide dismutase (SOD)
8. Glutathione peroxidase (GPx)
9. Catalase (CAT)
10. Malondialdehyde (MDA)
11. Nitric oxide (NO)
12. Auto antibody against oxidized LDL-C (ox-LDL ab)

Methods of evaluation

All the biochemical estimations are done by using computer assisted semiautomatic ‘Microlab 100’, ‘Microlab 200’ and ‘Mois Mini’. The results obtained are statistically analysed and compared between different groups of the study by applying standard statistical procedures to evaluate the notable changes or otherwise among different groups in the study.

METHODS OF ESTIMATION

ESTIMATION OF BLOOD GLUCOSE

Blood glucose is estimated by GOD-POD method. Commercially available reagent of MERCK is used for this purpose.

PRINCIPLE OF THE ASSAY

The intensity of the colour formed is proportional to the glucose concentration in the sample.

\[
\text{Oxidase} \\
\text{Glucose} + O_2 + H_2O \rightarrow \text{Gluconic acid} + H_2O_2
\]
Peroxidas

\[ 2 \text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{Phenol} \rightarrow \text{Quinoneimine} + 4\text{H}_2\text{O} \]

**REAGENTS**

Reagent 1: This reagent contains

- Phosphate buffer; pH 7.0 \(100\text{ mmol/l}\)
- Phenol \(5\text{ mmol/l}\)
- 4-Aminoantipyrine \(0.5\text{ mmol/l}\)
- Glucose Oxidase \(\geq 15\text{ LK/l}\)
- Peroxidase \(\geq 1\text{ KU/l}\)

Reagent 2: Standard

- Glucose \(100\text{ mg/dl}\)

**SPECIMEN**

Serum or EDTA plasma is taken for the performance of test.

**TEST PROCEDURE**

The assay conditions are

- Wavelength \(500\text{nm}\)
- Cuvette specification \(1\text{ cm light path}\)
- Temperature \(37^\circ\text{C}\)

Instrument is adjusted to zero with blank of reagent.

Reagent is pipetted into the cuvette marked Blank, Standard and Sample

<table>
<thead>
<tr>
<th>Working reagent (ml)</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Reagent ((\mu l))</td>
<td>-------</td>
<td>10</td>
<td>-------</td>
</tr>
<tr>
<td>Sample ((\mu l))</td>
<td>-------</td>
<td>-------</td>
<td>10</td>
</tr>
</tbody>
</table>
The whole set of tubes are allowed to equilibrate in an incubator for 15 minutes at
37°C.

After incubation the absorbance (A) of the sample and standard are read, against
the reagent blank. The colour is stable at least for 30 minutes. The procedure is
repeated three times with each standard.

**CALCULATION**

Glucose (mg/dl) = \( \frac{A_s}{A_{std}} \times \) concentration of standard.
\[ A_s = \text{Reading of sample} \]
\[ A_{std} = \text{Reading of standard} \]

**ESTIMATION OF HAEMOGLOBIN**

(Cyanmethaemoglobin Method)

**PRINCIPLE OF THE ASSAY**

Potassium ferricyanide converts hemoglobin in the sample to methaemoglobin.
Methaemoglobin further reacts with potassium cyanide to form a stable
cyanmethaemoglobin complex. Colour intensity of the complex formed is directly
proportional to the amount of hemoglobin present in the sample.

**REAGENT PREPARATION**

HEMOCOR – D, commercially available reagent is ready to use, which is protected
from exposure to bright light and kept tightly stoppered.

**SAMPLE MATERIAL**

Whole blood is collected in EDTA tube.

**PROCEDURE**

The assay conditions are:

- **Wavelength**: 540 nm
- **Temperature**: 24-28°C
- **Light path**: 1 cm
Pipetted into clean dry test tubes labeled as Blank (B), and Test (T)

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B (ml)</th>
<th>T (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEMOCOR - D Reagent</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Sample (well mixed whole blood)</td>
<td>----</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The contents of cuvette are mixed well and incubated at room temperature (25°C) for at least 5 min. The absorbance of the test sample (Abs.T) is measured against the reagent blank. The final colour is very stable.

**CALCULATION**

Haemoglobin in g/dl = Abs.T × 36.8

**ESTIMATION OF TOTAL CHOLESTEROL**

Serum total cholesterol is estimated by CHOD-PAP method. The assay is performed by using commercially available “LABKIT” reagent.

**PRINCIPLE OF THE ASSAY**

The series of reactions involved in the assay system is as follows.

- **Cholesterol Esters**
  - Cholesterol Esters + H₂O → Cholesterol + fatty acid
  - Oxidase
  - Cholesterol + O₂ → 4-cholestenone + H₂O₂
  - Peroxidase
  - 2 H₂O₂ + Phenol + 4-aminophenazone → Quinoneimine + 4 H₂O
The intensity of the colour formed is proportional to the cholesterol concentration in the sample.

**REAGENTS**

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Buffer)</td>
<td>Pipes pH 6.9</td>
<td>Cholesterol Oxidase (CHOD)</td>
</tr>
<tr>
<td>Phenol</td>
<td>26mmol/L</td>
<td>300 U/L</td>
</tr>
<tr>
<td>Cholesterol Esters (CHE)</td>
<td>300 U/L</td>
<td>Peroxidase (POD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1250 U/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-aminophenazone (4-AP)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4mmol/L</td>
</tr>
<tr>
<td>Cholesterol cal</td>
<td>Cholesterol calibrator</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 mg/ml</td>
</tr>
</tbody>
</table>

**REAGENT PREPARATION**

Working reagent: One vial of R2 (enzyme) is dissolved in the bottle R1 (buffer). After proper capping the reagent is shaken gently to mix contents. Working reagent is stable for at least 4 months at 2-8°C.

**SPECIMEN**

Serum is taken for the performance of the test.

**TEST PROCEDURE**

Assay conditions are

- **Wavelength**: 505 nm
- **Cuvette specification**: 1 cm light path
- **Temperature**: 37°C

Spectrophotometer is to zero with blank of reagent.

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Blank</th>
<th>Calibrator</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Calibrator (μl)</td>
<td>------</td>
<td>10</td>
<td>------</td>
</tr>
<tr>
<td>Sample (μl)</td>
<td>------</td>
<td>10</td>
<td>------</td>
</tr>
</tbody>
</table>
The content of all cuvettes are mixed well and incubated for 5 minutes at (15 - 25° C)
The absorbance of the test sample (Abs.T) and standard (Astd) are measured against the reagent blank. The colour is stable at least 30 minutes.

CALCULATION \( (\text{mg/dl}) = \frac{\text{Abs.T}}{\text{Astd}} \times 200 \)

ESTIMATION OF HDL CHOLESTEROL

HDL cholesterol is estimated by using commercially available reagent of Bayer Diagnostic Limited.

PRINCIPLE OF THE ASSAY

Chylomicron, VLDL cholesterol and LDL cholesterol fractions are separated from HDL by precipitating with Phosphotungstic Acid and MgCl₂. After centrifugation, cholesterol in HDL fraction that remains in the supernatant, is assayed by enzymatic cholesterol method.

CHE

\[ \text{Cholesterol Esters} + \text{H}_2\text{O} \quad \rightarrow \quad \text{Cholesterol} + \text{fatty acid} \]

CHO

\[ \text{Cholesterol} + \text{O}_2 \quad \rightarrow \quad 4\text{-cholestenone} + \text{H}_2\text{O}_2 \]

\[ 2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminophenazone} \quad \rightarrow \quad \text{Quinoneimine} + 4\text{H}_2\text{O} \]
The intensity of the colour formed was proportional to the HDL cholesterol concentration in the sample.

**REAGENTS**

R1 (Enzyme / Chromogen):
- Cholesterol Esters ≥200 U/L
- Cholesterol Oxidase ≥250 U/L
- Peroxidase ≥1000U/L
- 4-aminophenazone 0.5mmol/L

R2 (precipitating reagent):
- Phosphotungstic Acid 2.4mmol/L
- Magnesium Chloride 39mmol/L
- HDL Cholesterol calibrator 0.5g/L

**REAGENT PREPARATION**

Working reagent: One vial R2 (enzyme) is dissolved in one bottle of R1 (buffer). Then mixed gently to dissolve the contents. Working reagent is stable for 1 month at 2-8°C.

**SPECIMEN**
Serum is taken for the performance.

**TEST PROCEDURE**

**Precipitation**

Dispensed into centrifuge tube:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Precipitating reagent R2</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>
The sample and reagent are mixed well and centrifuged at 3500 rpm for 10 minutes. The clear supernatant is immediately separated and the cholesterol content in the specimen is determined.

**Assay conditions**

- Wavelength of the spectrophotometer used: 500nm
- Cuvette specification: 1 cm light path
- Temperature: 37° C

Spectrophotometer is adjusted to zero with reagent blank.

Pipetted into the cuvette

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Calibrator (μl)</td>
<td>------</td>
<td>20</td>
<td>------</td>
</tr>
<tr>
<td>Supernatant (μl)</td>
<td>------</td>
<td>--------</td>
<td>20</td>
</tr>
</tbody>
</table>

All the reagents and sample are mixed well and incubated for 5 minutes at 37° C. The absorbance of the sample (As) and standard (Astd) are read, against the blank. The colour is stable at least for 30 minutes.

**CALCULATION**

$$\text{HDL cholesterol (mg/dl)} = \left( \frac{A_s}{A_{std}} \right) \times \text{concentration of standard.}$$
ESTIMATION OF LDL CHOLESTEROL

PRINCIPLE OF THE ASSAY

Serum LDL cholesterol is estimated by a homogeneous method based on an innovative detergent technology. In the present study, commercially available kit of Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan is used.

When reagent 1 is mixed with a serum specimen as the first step, of the assay detergent 1 disrupts the structure of chylomicrons, VLDL-C and HDL-C, and causes release of cholesterol. The free cholesterol, transformed by cholesterol esterase, reacts with hydrogen peroxide-producing cholesterol oxidase. Hydrogen peroxide is consumed by a peroxidase in the presence of 4-aminoantipyrine to generate a colorless product. The second step of the assay started with the addition of reagent 2, releasing cholesterol from the remaining LDL-C, thereby allowed an enzymatic reaction to take place. Reagent 2 also (DSBmT), and hydrogen peroxide formed by the enzymatic reaction, produces a blue purple product. Intensity of coloration is proportional to concentration of LDL cholesterol.

REAGENTS

Reagent 1 : (Enzyme solution)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergent 1</td>
<td></td>
</tr>
<tr>
<td>4-Aminoantipyrine</td>
<td>0.5mml/l</td>
</tr>
<tr>
<td>Cholesterol Oxidase</td>
<td>1.2U/ml</td>
</tr>
<tr>
<td>Cholesterol esterase</td>
<td></td>
</tr>
<tr>
<td>Peroxidase</td>
<td></td>
</tr>
<tr>
<td>Good’s buffer solution</td>
<td>pH 6.3</td>
</tr>
</tbody>
</table>

Reagent 2 : (Colour reagent)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergent 2</td>
<td></td>
</tr>
<tr>
<td>N,N-bis(4-sulfobutyl)-m-tolune</td>
<td>1.0mml/l</td>
</tr>
<tr>
<td>Good’s buffer solution</td>
<td>pH 6.3</td>
</tr>
</tbody>
</table>
SPECIMEN
Serum is taken for the experiment.

TEST PROCEDURE
Assay conditions are

a. Wavelength of the spectrophotometer used 546nm
b. Cuvette specification 1 cm light path
c. Temperature 37° C

The instrument is adjusted to zero with blank of the reagent.

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent R-1 ((μl))</td>
<td>600</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>Calibrator (μl)</td>
<td>------</td>
<td>6</td>
<td>------</td>
</tr>
<tr>
<td>Sample (μl)</td>
<td>------</td>
<td>------</td>
<td>6</td>
</tr>
</tbody>
</table>

Reagents are mixed and incubated for 5 minutes at 37° C
Then pipetted 200μl of working reagent R 2 to each of Blank, Standard and Sample cuvettes
All the reagents and sample are mixed well and incubated for 5 minutes at 37° C.
The absorbance (A) of the sample (As) and Standard (Astd) are read against the blank. The colour is stable for at least 30 minutes.

CALCULATION
LDL cholesterol (mg/dl) = (As / Astd) x concentration of standard.
ESTIMATION OF MALONDIALDEHYDE (MDA)  

PRINCIPLE OF THE ASSAY

Assay of MDA using Thiobarbituric acid (TBA) is modified by Buege and Aust's, providing a simple, reproducible, and standardized tool for the assay of lipid peroxidation not only in serum, but also in urine, tissue homogenates, plasma and cell lysates. The MDA-TBA adducts formed by the reaction of MDA and TBA under high temperature (90-100°C) and in acidic condition is measured colorimetrically at 532 nm.

![Chemical Structure](image)

REAGENTS

1. 15% (W/V) Trichloracetic acid (TCA): 15 gm of TCA is added in water and volume is made to 100 ml.
2. 0.375% (W/V) Thiobarbituric Acid (TBA): 0.375 gm of thiobarbituric acid is added to water and volume is made to 100 ml. The solution is heated mildly and cooled before dilution.
3. 0.25 (N) HCL is prepared as normal procedure.
4. TCA-TBA-HCL solution: Reagent 1,2 and 3 are mixed in the ratio of 1:1:1, and prepared fresh before use.
5. n- butanol.
6. Preparation of working standard: 1,1,3,3-tetraethoxypropane (TEP) is used as the standard. A series of TEP working standard are prepared by diluting the
stock 100 μmol/L TEP standard with water. Absorbance of each standard is plotted against the corresponding concentration as shown in the graph.

**SAMPLE PREPARATION**
Venous blood samples are drawn in vacutainer in the morning after an overnight fast or in the case of myocardial infarction (MI), immediately after admission, mostly within one hour of MI. Smoking or heavy physical activity is not allowed in the morning before sample collection. Blood samples are immediately placed in ice. Fresh samples are analysed within 1 to 4 hours of sample collection. Blood is allowed to clot for 15 minutes, and then centrifuged at 3000 rpm for 10 minutes. The serum is separated and transferred to another dry labeled tube for estimation.

**TEST PROCEDURE**
a) 0.8 ml of serum is added to 1.2 ml of TBA-TCA-HCL reagent.
b) Serum and reagent are mixed well and kept in water bath at 100 °C for 15 minutes.
c) Then it is cooled immediately in ice chilled water.
d) 2 ml of n-butanol is added to it and mixed well with vortex and centrifuged for 5 minutes at 2000 rpm.
e) Pink colored supernatant is taken for measurement at 532nm.

**STANDARD CURVE OF TOTAL MALONDIALDEHYDE CONCENTRATION**
1. Calculation of malondialdehyde concentration Is done by the end-point procedure proved straightforward and adequate for most samples. Worked with a time point in which the readings of the absorbances lie within the linear range in the curve.
2. The average of the triplicate absorbance readings are taken for each standard, sample and blank.

3. The average absorbance value of the blank is subtracted from that of each standard and sample.

4. Net absorbance values of each standard is plotted against number of µmol/L of MDA. This is standard curve for MDA (Fig III-1).

5. MDA concentration is then calculated for each sample from the linear part of the standard curve.

Fig.III-1 MDA standard curve

Linear graph paper is used and plotted the mean MDA equivalents for each standard used on the X axis against the corresponding spectrophotometer readings on the Y axis. The concentration of MDA equivalents in µmol/L in specimen is determined by interpolation from the standard curve.
**CALCULATION**

1. The standard data are used and calculated the net $A_{532}$ for each standard by subtracting the blank ($A_0$) value from each of the standard $A_{532}$ values. The net $A_{532}$ against $[MDA]$ is plotted, and performed a linear regression analysis of $A_{532}$ on $[MDA]$:

$$[MDA] = a[MDA] + b$$

2. The concentration of analyte is calculated in each unknown sample from the net $A_{532}$ of the sample:

$$[MDA] = \frac{A_{532} - b}{a} \cdot df$$

where:
- $[MDA]$ = the concentration of MDA in $\mu$mol of the sample
- $A_{532}$ = net absorbance at 532 nm of the sample
- $a$ = regression coefficient (slope)
- $b$ = intercept
- $df$ = dilution factor

**PRECISION**

When a series of ten human serum samples are assayed on the same day, the intra-assay coefficient of variation amounts to 5.5%. But for eight human serum samples on seven different days under the same experimental conditions, the intra-assay coefficient of variation is found to be 5.9%.
ESTIMATION OF NITRIC OXIDE

PRINCIPLE OF THE ASSAY

Griess Reagent System is based on the chemical reaction shown here, which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride under acidic conditions. In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. Spectrophotometric quantitation of nitrite using Griess Reagent is straightforward. This method employs metallic cadmium for quantitative conversion of nitrate to nitrite prior to quantitation of nitrate by the use of Griess reagent.

Fig. III 2: Chemical reactions involved in the measurement of NO\textsuperscript{-} using the Griess Reagent System
**PREPARATION OF REAGENTS**

a) **Granular Cadmium**: Granulated cadmium beads are used for nitrate reduction.

b) **Trichloroacetic Acid (20%)**: 20% (wt/vol) Trichloroacetic acid is used for sample deproteination.

c) **Color Reagent 1**: Sulfanilamide – 1% (p-Aminobenzenesulfonamide) is dissolved in 3N HCl.

d) **Color Reagent 2**: 0.030 gm of N- (1-Naphthyl) ethylenediamine dihydrochloride is dissolved in 3 ml of deionized H2O.

e) **Griess Reagents**: Color reagent 1 and color reagent 2 are mixed in the ratio of 1:1 before assay.

**PREPARATION OF STANDARD CURVE**

Preparation of working standard: Potassium nitrate is used as the standard. A series of potassium nitrate working standard containing 1,2,3,4,5,10,15,20,25 and 30 μmol/L are prepared by diluting the stock 100 μmol/L potassium nitrate standard with water. Absorbance of each standard is plotted against the corresponding concentration as shown in the graph.

**SAMPLE PREPARATION AND ASSAY**

1. 1 ml clear unhaemolysed serum is taken in a new dry test tube.

2. 1 ml of 20% (wt/vol) TCA is added and vigorously mixed and is incubated at room temperature for 15 minutes.

3. Then the sample is centrifuged at 3,000 rpm for 5 minutes.

4. The resulting supernatant was transferred to a fresh dry test tube.

5. Approximately 1-2 gm of dried granular Cd beads are added in the tube.

6. The sample and Cd mixture are incubated for overnight at room temperature.
7. After incubation, the sample is transferred to a clean test tube and centrifuged for 5 minutes at 3,000 rpm and then it is ready for assay to be done within one hour.

8. In a clean dry test tube Cd treated 0.5 ml of clear sample is taken and 0.5 ml of Griess reagent is mixed with it.

9. Then the whole mixture is incubated for 5–10 minutes at room temperature protected from light. A purple/magenta color forms immediately.

10. After incubation, the absorbance value is read at 540 nm.

![Absorbance spectrum of the colored azo compound](image)

**Fig. III 3: Absorbance spectrum of the colored azo compound**

**CALCULATION**

**Subtraction of the blanks**

The mean absorbance value of the blank is subtracted from the absorbance values of the entire test.

Sample concentration of nitric oxide is determined by using Y-intercept equation as demonstrated below.

1. The mean O.D. values for each replicate of sample and standard are taken.
2. The standard curve is prepared by using the standard concentration (X-axis) vs. the corresponding O.D. (Y-axis).
3. The concentration of each sample is estimated by interpolation from the standard curve using the Y-intercept equation. A sample O.D. value is entered in place of “Y” and solved for “X” to determine the concentration.
Y=mX+b, where
Y= the ordinate or y-axis value (corresponds to the OD reading).
X= the abscissa or x-axis value (corresponds to the NO concentration).
m= slope.
b= Y-axis intercept.

A linear graph paper is used; mean NO equivalents are plotted for each standard on the X axis against the corresponding spectrophotometer reading on the Y axis. The concentration of NO equivalents in µmol/L are determined in specimens by interpolation from the standard curve.

ESTIMATION OF AUTOANTIBODIES AGAINST OXIDIZED LOW DENSITY LIPOPROTEIN CHOLESTEROL

PRINCIPLE OF THE ASSAY

Autoantibodies against ox-LDL-C are measured according to the modified method of Lehtimaki et al. Estimation of auto antibody against oxidized LDL-C is an
enzymatic immunoassay, designed to determine human autoantibodies against oxidized LDL-C directly in serum. Cu\(^{++}\) oxidized LDL-C is coated into micro titer plate as an antigen. Auto antibodies, if present in the serum, bind specifically to the antigen. After a washing step, a specific peroxidase conjugated anti human monoclonal antibody detects the presence of bound auto antibodies. After removal of unbound conjugate through extensive washing, o-phenylenediamine is added to the wells as a chromogenic substrate. The concentration of specific auto antibodies in the sample are quantified by a standard ELISA reader. The amount of color developed is directly proportional to the concentration of auto antibodies present in the sample.

**REAGENTS**

1. **EDTA (0.27 mmol/L)**: 0.27 mmol EDTA is mixed in 1000 ml of phosphate buffer saline (pH – 7.4)
2. **Butylated hydroxytoluene (BHT) 20 µmol/L**: 20 µmol BHT is diluted in 1000 ml of DMSO solution.
3. **CuSO\(_4\) (2 µmol/L)**: 2 µmol of CuSO\(_4\) is dissolved in 1000 ml of water.
4. **Phosphate buffer saline (PBS) (pH – 7.4)**: PBS (pH – 7.4) was ready for use.
5. **Washing Buffer**: 0.5 ml of Tween 20 is added with 100 ml of PBS and mixed well, then again 100 ml of water is added and mixed thoroughly.
6. **Tween 20**
7. **IgG peroxidase**: IgG peroxidase conjugated rabbit antihuman monoclonal antibody (No. 55220 Cappel, Organon) is diluted 1:4000 (vol/vol) in buffer. The buffer contained 0.27 mmol EDTA, 1% BHT and 0.05% Tween 20.
8. **o-phenylenediamine (Sigma) (0.4 mg/ml)**: o-phenylenediamine is used as color reagent.
9. **H\(_2\)O\(_2\) (0.045%)**: 0.045% H\(_2\)O\(_2\) is prepared as normal procedure.
10. **H\(_2\)SO\(_4\) (2M)**: 2M H\(_2\)SO\(_4\) is prepared as normal procedure and used as a stopping solution.

**TEST PROCEDURE**
1. Native LDL is protected against oxidation by 0.27 mmol/L EDTA and 20 μmol/L butylated hydroxy-toluene (BHT) in PBS. Oxidized LDL is obtained after 24-hour oxidation of the native LDL with 2 μmol/L CuSO₄.

2. For the antigen coating, half of the wells are coated with 50 μL of native antigen on a polystyrene plate (Nunc). The other half is coated with 50 μL copper-oxidized LDL antigen (both at a concentration of 5 μg/ml) and is incubated for 16 hours at 4°C.

3. Unbound antigen is removed by washing of the wells. The remaining nonspecific binding sites are saturated by using 2% human serum albumin in PBS and 20 μmol/L BHT and incubated for 2 hours at 4°C. After incubation the plates are washed thoroughly using washing buffer and 50 μL of the serum samples (diluted 1:20) are added to the wells coated with native and oxidized LDL and again are incubated overnight at 4°C.

4. After the incubation, the wells are aspirated and washed 6 times with washing buffer before adding of IgG-peroxidase-conjugated rabbit anti-human monoclonal antibody (No. 55220 Cappel, Organon), which is diluted 1:4000 (vol/vol) in buffer (0.27 mmol/L EDTA, 20 μmol/L BHT, 1% BSA, 0.05% Tween 20), and then incubated for 4 hours at 4°C.

5. After incubation the wells are washed with washing buffer thoroughly and added 50 μL of freshly prepared substrate (0.4 mg/mL o-phenylenediamine and 0.045% H₂O₂ in 100 mmol/L acetate buffer, pH 5.0) is added in wells and are incubated for exactly 5 minutes at room temperature.

6. The enzyme reaction is terminated by adding 50 μL of 2 M H₂SO₄. The optical density (OD) is measured at 492 nm using an ELISA reader.

**CALCULATION OF RESULTS**

A standard curve is prepared from the standards. Results of the samples are read from the standard curve.
ESTIMATION OF SUPEROXIDE DISMUTASES (SOD)\textsuperscript{10}

**PRINCIPLE**

The assay procedure involves the inhibition of Epinephrine auto oxidation in an alkaline medium (pH 10.2). The reaction is started by adding the epinephrine solution to the assay mixture, containing blood hemolysate, sodium salt of EDTA and sodium carbonate, and the change in extinction coefficient is followed at 480 nm in spectrophotometer. The rate change of extinction as reported is 0.025/minute at 25°C. Enzyme activity is expressed in arbitrary units considering 50\% inhibition in the reaction mixture under the experimental conditions as one unit of SOD.

**Preparation of reagents**

1. **Epinephrine:** Fresh 10 mM Epinephrine is prepared in dilute HCL pH 2.0 and is covered with tin foil.

2. **EDTA:** 10mM EDTA solution is prepared freshly.

3. **Sodium Carbonate:** 75 mM carbonate solution is prepared and pH is adjusted at 10.2.
4. **Preparation of working standard**: A series of SOD working standard are prepared by diluting the stock 3000 U/ml standard with water. Absorbance of each standard was plotted against the corresponding concentration as shown in the graph.

**SAMPLE PREPARATION**

a) 0.5 ml of heparinised blood is collected in a fresh dry tube and centrifuged at 3000 rpm for 5 minutes. The whole plasma is discarded from the sample without disturbing buffy coat.

b) Remaining red cells are washed thrice by adding normal saline and centrifuged.

c) To the washed cells, 4.0 ml chilled distilled water is added and mixed well.

d) Then 1.0 ml chilled ethanoll and 0.6 ml of ice cold chloroform is added in tube and mixed well.

e) The tube is incubated for 5 minutes at 4°C and then centrifuged for 5 minutes at 3000 rpm at 4°C.

**ASSAY PROCEDURE**

*Assay conditions are*

- Wavelength of the spectrophotometer: 480 nm
- Cuvette specification: 1 cm light path
- Temperature: +25°C

The spectrophotometer is adjusted to zero with water as blank.

Cuvettes are arranged as Blank, Standard and Sample

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (μl)</td>
<td>700</td>
<td>700</td>
<td>700</td>
</tr>
<tr>
<td>75mM sodium carbonate (μl)</td>
<td>2000</td>
<td>2000</td>
<td>2000</td>
</tr>
<tr>
<td>10 mM EDTA (μl)</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Blood sample (μl)</td>
<td>------</td>
<td>--------</td>
<td>50</td>
</tr>
</tbody>
</table>
The contents of all cuvetts are well mixed followed by addition of 180 µl of 10 nM Epinephrine to each of them. Readings are taken immediately after adding of epinephrine in every 30 seconds interval till the rate change of extinction is of 0.025/minute.

**CALCULATION OF RESULTS**

Results of the samples are read from the equation.

\[
\text{SOD (U/ml)} = \frac{A_s}{A_{std}} \times \text{Cont. of Std}
\]

**ESTIMATION OF GLUTATHIONE PEROXIDASE (GPx)**

**PRINCIPLE**

A known amount of enzyme is allowed to react with H$_2$O$_2$ and GSH for a specific time period. After the reaction the remaining GSH content in the solution is measured by Ellman’s reaction.

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]
REAGENTS

a) Sodium Phosphate Buffer-0.32M, pH – 7.0 :
   Working Solution: 4.23 gm of \( Na_2HPO_4 \) and 4.1 gm of \( NaH_2PO_4 \cdot 2H_2O \) are taken in cylinder and the volume made up to 100 ml with water and adjusted at pH-7.0

b) EDTA- 2mM
   Working Solution: 0.045 gm of EDTA is taken in 10 ml of water

c) DTNB-0.5mM
   Working Solution: 0.0020 gm of DTNB is taken in 10 ml of PO\(_4\) buffer

d) Sodium Azide-10 nM
   Working Solution: 0.0065 gm of sodium azide is taken in 10 ml of water

e) \( H_2O_2 \) – 0.25mM
   Working Solution: Freshly prepared 2\( \mu \)l 30% \( H_2O_2 \) is added in 8.6 ml of water

f) Reduced glutathione- 4mM
   Working Solution: 0.0120 gm of reduced glutathione is added in 10 ml PO\(_4\) buffer

g) TCA – 10% Solution
   Working Solution: 10 gm of TCA is taken in cylinder and the volume made up to 100 ml with water.

SAMPLE PREPARATION:

a) 0.5 ml of heparinised blood is collected in a fresh dry tube and centrifuged at 3000 rpm for 5 minutes. The whole plasma is discarded from the sample without disturbing buffy coat.

b) Remaining red cells are washed thrice with normal saline.
c) After cell washing, 4.6 ml chilled distilled water is added and mixed well.
d) Then 1.0 ml chilled ethanol and 0.6 ml of ice cold chloroform are added to tube and mixed well.
e) The tube is incubated for 5 minutes at 4°C and then centrifuged for 5 minutes at 3000 rpm at 4°C. The clear supernatant is used as test sample.

ASSAY PROCEDURE

Pipetted into the cuvette marked Test, Control and Standard the following reagents as tabulated below:

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>TEST (µL)</th>
<th>CONTROL (µL)</th>
<th>STANDARD (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Phosphate Buffer</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>GSH - 4 mM</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EDTA - 2mM</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sodium Azide - ηM</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>H₂O₂ - 0.25mM</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10% TCA</td>
<td>--------</td>
<td>500</td>
<td>--------</td>
</tr>
<tr>
<td>Hemolysate</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Distilled water</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
</tbody>
</table>

All the cuvettes are incubated at 37°C for 10 minutes. Then immediately 500 of µL 10% TCA is added in the test and standard tube to stop the reaction.

| 10% TCA | 500 | -------- | 500 |

All the cuvettes are centrifuged at 3000 rpm for 5 minutes and the supernatants are taken for the assay.
All the cuvettes are incubated at room temperature for 10 minutes. Elman's reaction is complete. Absorbance of the color products is measured at 412 nm.

**ESTIMATION OF HEMOGLOBIN**

Hemoglobin (gm%) is estimated of the same sample by the method as describe earlier.

**STANDARD CURVE FOR GLUTATHIONE PEROXIDASE:**

![Standard curve of GPx](image)

**CALCULATION**

Total Glutathione peroxidase (µmol/g Hb%) = µ mole glutathione utilized / Hb gm%
ESTIMATION OF CATALASE

BACKGROUND

PRINCIPLE

In the ultraviolet range $H_2O_2$ shows a continued increase in absorption with decreasing wavelength. Decomposition of $H_2O_2$ by catalase can be followed by the decrease in extinction at 240 nm and the difference in extinction per unit time is a measure of catalase activity.

\[
\text{(Catalytic Activity)} \quad 2H_2O_2 \xrightleftharpoons{\text{Catalase}} O_2 + 2H_2O
\]

\[
\text{(Peroxidatic Activity)} \quad H_2O_2 + AH_2 \xrightarrow{\text{Catalase}} A + 2H_2O
\]

REAGENTS

Preparation of 50 mm Phosphate buffer solution pH 7.0

a) $KH_2PO_4$ solution: 6.81gm of $KH_2PO_4$ is dissolved in distilled water and the volume made up to 1000 ml to prepare the solution (a).

b) $K_2HPO_4$ solution: 11.41gm of $K_2HPO_4$ is dissolved in distilled water and volume is made up to 1000 ml to prepare the solution (b).

Solution (a) is mixed with solution (b) until the pH-7.0 attain.

Preparation of $H_2O_2$ buffer solution

The solution is prepared by adding 0.06 ml of 30% $H_2O_2$ to 50 ml of phosphate buffer and the optical density of the solution is adjusted to $0.500 \pm 0.010$ at 240 nm by adding $H_2O_2$ or phosphate buffer, as required.
**SPECIMEN**

Whole blood haemolysate (as described earlier in GPx estimation) is taken for the performance.

**TEST PROCEDURE**

**Assay Conditions**

- **d.** Wavelength of the spectrophotometer: 240nm
- **e.** Cuvette specification: 1 cm light path
- **f.** Temperature: 25°C
- **g.** Delay time: 30 sec.
- **h.** Interval time: 15 sec.
- **i.** No of reading: 16
- **j.** $E_{240\text{nm}}$: 0.040 [cm$^2$/μmole]

The spectrophotometer is adjusted to zero with blank of buffer.

Pipetted into the cuvette as specified below.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent (ml)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Sample (μl)</td>
<td>------</td>
<td>20</td>
</tr>
</tbody>
</table>

The reagent and sample are mixed well and incubated for 30 seconds at 25°C. Then the reading is taken immediately and changes of the absorbance is noted in seconds still the OD decreases from 0.0450 to 0.0400.

**Calculation U/ml) = 17 \times 13.1 \times 0.02 / Time (sec)**
REFERENCE


