CHAPTER IV

MATERIALS AND METHODS
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The subjects for the present study were selected from Sahakarana Hrudayalaya, Academy of Medical Sciences, Pariyaram, Kannur district of Kerala state during the years 2006 to 2012. Those in whom a diagnosis of myocardial infarction was confirmed clinically, electrocardiographically and biochemically (elevated cardiac enzymes) were selected for the study. Subject groups with previous history of MI, stroke, heart disease, liver disease, blood disorders, type 1 diabetes, and other co-morbid illnesses were excluded. Also, patients with risk factors other than smoking, diabetes, hypertension, and dyslipidemia were excluded, since they are not major concern in Indian population.

Apparently healthy subjects without any previous history of chest pain or myocardial infarction (MI) served as controls. Data collected from the controls were similar to those collected from the patients. We have selected 606 cases and 615 age and sex matched controls. In test group there were 528 (87.1 %) males and 78 (12.9 %) females. Similarly in control group 535 (87.0 %) were males and 80 (13.0%) were females. Thus a total of 1221 subjects, of which 87.0% were males and 13.0% were females.

The demographic data collected from each case included age, sex, education, dietary habit, physical exercise, job, income and history of
diabetes, hypertension and smoking. Only patients with complete
demographic data were included in the study.

A written consent was obtained from them or their relatives who
accompanied them after explaining the details of the study. Five millilitre
venous blood was also collected from each person by venipuncture, of which
one millilitre was collected in fluoride oxalate bottle, one millilitre in buffered
sodium citrate (3.2%) containing bottle and the remaining three ml in plain
dry tube. Plasma separated from anticoagulated blood samples were used for
the estimation of glucose and fibrinogen. The blood collected in dry tube was
allowed to clot and serum was separated. This serum sample was used for all
the biochemical investigations in this study. As far as possible the
investigations are carried out on the same day of collection or the samples
were stored at -70 °C. All the reagents and test kits for the quantitative
estimation were obtained from Merck group India and Transasia biomedical
company, Mumbai.

The investigations were analysed using semi automated clinical
chemistry analyser micro lab 300 by Merck group India, fully automated
clinical biochemistry analysers Roch Cobas C311 by Roch Diagnosis Pvt
Ltd, Mumbai and Beckman Coulter AU 680 by Beckman Coulter India Pvt
Ltd, Mumbai.
Using the serum sample, the following biochemical investigations were done:

1. Cholesterol by cholesterol oxidase-peroxidase method.
2. LDL cholesterol by turbidimetric immunoassay.
3. HDL cholesterol by turbidimetric immunoassay.
4. Triglycerides by glycerol phosphate oxidase (GPO) – Trinder method.
5. Lipoprotein (a) by multipoint calibration with fixed time mode.
6. Aspartate aminotransferase (AST) by kinetic method.
7. Creatine phosphokinase-MB by Immunoinhibition method.
8. Troponin T by electrochemiluminescence immunoassay (ECLIA).
9. Troponin I by immunoassay homocysteine by Enzyme Immunoassay (EIA).
10. C - reactive protein by ELISA method.

Using the plasma sample, the following biochemical investigations were done:

1. Fibrinogen was estimated by immunochemical method.
2. Glucose was estimated by oxidase/ peroxidase method.
4.1. Fasting Lipid Profile:

4.1.1. Estimation of cholesterol by cholesterol oxidase-peroxidase method:

**Principle of the assay:** - The estimation of cholesterol involves the following enzyme catalyzed reactions. Serum sample is treated with cholesterol esterase and cholesterol oxidase to get free cholesterol and fatty acids. Oxidation of free cholesterol by enzyme cholesterol oxidase liberates hydrogen peroxide, which is treated with enzyme peroxidase to get nascent oxygen. This reacts with chromogen and gives a coloured complex.

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

\[ \text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestenone} + H_2O_2 \]

\[ 2 H_2O_2 \xrightarrow{\text{Peroxidase}} 4 H_2O + O_2 \]

\[ \text{Aminoantipyrine} + \text{Phenol} + O_2 \xrightarrow{} \text{Quinonimine} \]

Absorbance of quinonimine so formed is directly proportional to the cholesterol concentration.

**Reagent composition:**
Cholesterol reagent: It contains cholesterol esterase 300 U/l, cholesterol oxidase 300 U/l, peroxidase 1250 U/l, 4-aminoantipyrine 0.4 mMol/l, acetate buffer 90 mMol/l and phenol 26 mMol/l.

**Procedure:**

<table>
<thead>
<tr>
<th>Contents added</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The contents in each tube is mixed well and kept at 37°C for five minutes. The absorbance of the standard and test is read at 505 nm against reagent blank.

**Calculation:**

\[
\text{Cholesterol mg/dL} = \frac{\text{Reading of sample} - \text{Reading of blank}}{\text{Reading of Standard} - \text{Reading of blank}} \times \text{Std Conc}
\]

**4.1.2. Estimation of LDL cholesterol by turbidimetric immunoassay:**

**Principle of the assay:**

At first polyanions and amphoteric surfactants protect LDL from enzyme reactions. The enzymes cholesterol esterase (CHE) and cholesterol oxidase (CO) added react with non LDL lipoproteins. Hydrogen peroxide
produced by the enzyme reactions with non LDL cholesterol is decomposed to water by the enzyme catalase. Again CHE and CO are added, which react only with LDL-C. Hydrogen peroxide produced by the enzyme reactions with LDL-C, yield a blue colored complex upon oxidase condensation with HDAOS (N-ethyl-N-2-hydroxy, 3-sulphopropyl, 3, 5-dimethoxy-4-fluoroaniline, sodium salt) and 4-aminoantipyrine (4AA) in the presence of peroxidase. By measuring the absorbance of the blue colour complex produced at the optimum wavelength of 600 nm the LDL-C concentration in the samples can be calculated when compared with the absorbance of the LDL-C calibrator.

1st Reaction

\[
\text{Chylomicrons + VLDL and HDL Cholesterol + O}_2 + \text{H}_2\text{O} \xrightarrow{\text{CHE and CO}} 4\text{–Cholestenone + Fatty acids + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} \text{O}_2
\]

2nd Reaction

\[
\text{LDL Cholesterol + H}_2\text{O} + \text{O} \xrightarrow{\text{CHE and CO}} 4\text{–Cholestenone + Fatty Acids + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{–Aminoantipyrine + HDAOS} \xrightarrow{\text{Peroxidase}} \text{Blue colour complex + 3H}_2\text{O}
\]

Reagent composition:
**Reagent 1:** It contains Goods buffer (pH 6.8) 25 mMol/l, HDAOS 0.64 mMol/l, cholesterol oxidase 5,000 U/l, catalase 10,000 U/l, ascorbate oxidase 5000 U/l.

**Reagent 2:** Goods buffer pH (7.1) 25 mMol/l, 4-aminoantipyrine 3.4 mMol/l, peroxidase 20,000 U/l.

Reagent 1 and reagent 2 are ready to use.

**Calibrator:** Accurately 3.0 ml of distilled water was added to dissolve the contents of each of the calibrator.

**Procedure:**

Sample/Control/Test: 220 microlitre reagent 1 was mixed with 3 microlitre sample and incubated for 5 minutes at 37 °C. The initial absorbance A1 was read and 90 microlitre of reagent 2 was added and incubated for 5 minutes and the final absorbance A2 was read after 5 minutes at 600 nm.

**Calculation:**

\[
\text{LDL} = \frac{C_{\text{calibrator of ion concentration}} \times (A_{1} - A_{2})}{C_{\text{calibrator of test A2}}} - 4.1.3. \text{Estimation of HDL cholesterol by turbidimetric immunoassay:}
\]

**Principle of the assay:**

79
Anti human beta lipoprotein antibody in reagent 1 binds to lipoproteins other than HDL. The formed antigen antibody complexes block enzyme reactions when reagent 2 is added. Cholesterol oxidase and cholesterol esterase in reagent 2 react only with HDL-cholesterol. Hydrogen peroxide produced by the enzyme reacts with HDL-cholesterol, \( \text{H}_2\text{O}_2 \) reacts with HDAOS and 4-aminoantipyrine in the presence of enzyme peroxidase to form blue colored complex. By measuring the absorbance of the blue colour complex produced at the optimum wavelength of 593 nm, the HDL-cholesterol concentration in the samples can be calculated when compared with the absorbance of the HDL-cholesterol calibrator.

Chylomicrons, VLDL & LDL – cholesterol → \text{Anti lipo antibody} → \text{Antigen antibody complex}

\[
\text{HDL cholesterol} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{CHE and CO}} \text{Cholestenone} + \text{H}_2\text{O}_2 + \text{Fatty acids}
\]

\[
2\text{H}_2\text{O}_2 + 4\text{– Aminoantipyrine} + \text{HDAOS} \xrightarrow{\text{Peroxidase}} \text{Blue colored complex} + 3\text{H}_2\text{O}
\]

**Reagent composition:**

**Reagent 1:** Goods buffer (pH 7) 30 mMol/l, 4 aminoantipyrine 0.9 mMol/l, peroxidase 24,000 U/l, ascorbate oxidase 2,700 U/l.

**Reagent 2:** Goods buffer (pH 7) 30 mMol/l, HDAOS 0.8 mMol/l,
Cholesterol oxidase 20,000 U/l, CHE 4,000 U/l
Calibrator:

Accurately 3.0 ml of distilled water was added to dissolve the contents of each of the calibrator.

Procedure:

Sample/Control/Test: 220 microlitre reagent 1 was mixed with 3 microlitre sample and incubated for 5 minutes at 37 °C. The initial absorbance A1 was read and 90 microlitre of reagent 2 was added and incubated for 5 minutes and the final absorbance A2 was read after 5 minutes at 600 nm.

Calculation:

\[
\text{Calibrator of ion concentration} = \frac{A_2 - A_1}{d l/mg HDL}
\]

4.1.4. Estimation of Triglycerides by Glycerol phosphate oxidase (GPO) – Trinder method:

Principle of the assay: Enzymatic methods for the estimation of triglycerides have been developed and are generally preferred over the older colorimetric method. Measurement of triglyceride is based on enzymatic hydrolysis of triglycerides to glycerol and fatty acids, with subsequent addition of liberated glycerol with ATP and glycerokinase to form ADP and glycerol phosphate. Glycerol phosphate undergoes oxidation to get H$_2$O$_2$.
treating with enzyme peroxidase to get nascent oxygen. It reacts with chromogens giving a coloured complex. It is then used to calculate the triglyceride concentration. The reaction sequence is as follows.

$$\text{Triglyceride} + \text{H}_2\text{O} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Free fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3phosphate} + \text{ADP}$$

$$\text{Glycerol-3 phosphate} + \text{O}^- + \text{O}_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxyacetone phosphate} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + 4 \text{Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 2 \text{H}_2\text{O}$$

Absorbance of quinoneimine so formed is directly proportional to the triglyceride concentration.

**Reagent composition:**

Triglyceride reagent: It contains lipase, glycerol kinase, glycerol phosphate, oxidase and peroxidase.

**Assay procedure:**

<table>
<thead>
<tr>
<th>Contents added</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
The contents in each tube is mixed well and kept at 37 °C for six minutes. The absorbance of the standard and test were read at 505 nm against reagent blank.

**Calculation:**

\[
\text{Triglycerides mg/dl} = \frac{\text{Reading of sample} - \text{Reading of blank}}{\text{Reading of Standard} - \text{Reading of blank}} \times \text{Std Conc}
\]

**4.2. Emerging risk factors**

**4.2.1. Estimation of lipoprotein (a) by multipoint calibration with fixed time mode.**

**Principle of the assay:** Latex particle coated with antihuman Lp (a) are agglutinated when mixed with samples containing Lp(a). The agglutination causes an absorbance change depending upon the Lp (a) content of the patient sample, that can be interpolated in a calibration curve prepared with different calibrators of different Lp (a) contents.

**Reagent composition:**

Reagent 1: Buffer solution (pH 8.3)

Reagent 2: Lipoprotein (a) latex

Lipoprotein (a) calibrator
Reagent 1 and 2 are ready to use, should gently be mixed before use.

The calibrator was reconstituted with 1 ml of distilled water.

Calibration curve

Prepare dilution of the Lp (a) calibrator using normal saline as diluent.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator, μl</td>
<td>0</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Normal saline, μl</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Dilution factor</td>
<td>0</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Multiply the Lp (a) calibrator concentration by the corresponding dilution factor indicated in the table to obtain the Lp (a) concentration of the different calibrators.

Procedure:

<table>
<thead>
<tr>
<th>Contents added</th>
<th>Calibrator</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator</td>
<td>3.75 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>3.75 µl</td>
</tr>
<tr>
<td>R1 buffer</td>
<td>200 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>R 2 latex</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
The absorbance was read against blank after 10 seconds (A1) and after 4 minutes (A2) after the latex addition.

**Calculation:**

The difference in the absorbance (A2-A1) was calculated of each diluted Lp (a) calibrator and the values found against the Lp (a) concentration in the sample is calculated by the interpolation of (A2-A1) value on the calibration curve.

**4.2.2. Determination of homocysteine by Enzyme ImmunoAssay (EIA)**

**Principle of the assay:** Homocysteine is circulates mostly in its oxidized forms bound to plasma proteins. Smaller amounts of reduced homocysteine and disulfide homocysteine (Hcy & SS-Hcy) are present in plasma. Total homocysteine represents the sum of all Hcy species found in plasma and serum. (Free plus protein bound).

The Enzyme ImmunoAssay (EIA) is intended for the quantitative determination of total L- Homocysteine in human serum or plasma. Protein bound homocysteine is reduced to free homocysteine and enzymatically converted to S-adenosyl homocysteine (SAH) in separate procedure prior to the immuno assay. The enzyme is specific for the L form of homocysteine, which is the only form present in the blood. Hcy, mixed disulfide and
protein-bound forms of Hcy in the sample are reduced to free Hcy by use of dithiotheritol (DTT).
Prot-SS-Hcy

*R1-SS-Hcy

Hcy –SS-Hcy

*R1 any thiol residue.

Enzymatic conversion: Hcy in the sample is converted to S-adenosyl-L-homocysteine by the use of SAH hydrolase and excess adenosine (Ad)

\[ \text{Hcy} + \text{Ad} \xrightarrow{\text{SAH hydrolase}} \text{SAH} + \text{H}_2\text{O} \]

The following solid phase enzyme immunoassay is based on competition between SAH in the sample and immobilized SAH bound to the walls of microtitre plate for binding sites on a monoclonal anti-SAH antibody. After removal of unbound anti-SAH antibody, a secondary rabbit anti-mouse antibody labeled with the enzyme horse radish peroxidase (HRP) is added. The peroxidase activity is measured spectrophotometrically after addition of substrate, and the absorbance is inversely related to the concentration of Hcy in the sample.

**Reagents:**


Reagent B (Adenosine/DTT solution): adenosine/ dithiotheritol, ctri acid.
Reagent C (SAH hydrolase): Recombinant S-adenosyl-L-homocysteine hydrolase, trisbuffer, glycerol, methylparaben

Reagent D (Enzyme inhibitor): Merthiolate, phosphate buffer.

Reagent E (Adenosine deaminase): adenosine deaminase, phosphate buffer, sodium azide, BSA (Bovine serum albumin), phenol red dye.


Reagent G (Enzyme conjugate): rabbit anti-mouse antibody enzyme conjugate, BSA, horse radish peroxidase, blue dye.


Reagent S (Stop solution): 0.8M Sulphuric acid.

Buff Wash (Wash buffer): phosphate buffer, Merthiolate, Tween 20, BSA.1/10 dilution before use with purified water.

Cal1-Cal6. (Calibrators): S-adenosyl-L-homocysteine (2, 4, 8, 15, 30, 50 µmol/L) in buffer with preservative.

Controls: Low, Medium and High controls (with a concentration of 7.0, 12.5, and 25.0 µmol/L homocystein in diluted serum sample of human origin, phosphate buffer and preservative.

Microtitre strips coated with S-adenosyl-L-homocysteine.
Procedure:

All solutions and microtitre strips are equilibrated to room temperature before use.

- **Sample pre-treatment procedure:** Sample pre-treatment solution must be made no more than one hour prior to the start of the assay. Mix 45ml of Reagent A, 2.5ml of Reagent B and 2.5ml of Reagent C. for 100 samples (no dead volume calculated).

- **Dilute calibrators and sample/controls** - 25µl calibrators and sample/controls+500 µl Sample pre-treatment solution. Mix well and incubate for 30min at 37 °C (cap the tubes or cover with parafilm during incubation).

Note: proceed with next step before the samples have cooled.

- Add 500 µl Reagent D. Mix well and incubate for 15min at 18-25°C.

- Add 500 µl Reagent E. Mix well and incubate for 5min at 18-25°C.

- Pipette 25 µl diluted calibrator and sample/control from step 4 in to the wells of SAH- coated microtitre strips.

- Add 200 µl Reagent F to each well. Mix well and incubate for 30min at 18-25°C. Use the enclosed lid during all incubations.
• Wash with diluted wash buffer. After washing empty the wells on paper towels.

• Add 100 µl Reagent G to each well. Incubate for 20min at 18-25°C.

• Wash with diluted wash buffer. After washing empty the wells on paper towels.

• Add 100 µl Reagent H to each well. Incubate for 10min at 18-25°C.

• Add 100 µl Reagent S to each well.

• Shake and read at 450nm within 15minutes.

4.2.3. Determination of C - reactive protein (CRP) by ELISA method

**Principle of the assay:** The CRP ELISA kit is a solid phase direct sandwich method. The samples and anti-CRP-HRP conjugate are added to the microwells coated with antibody to CRP (MAb). CRP in the patient’s serum binds to anti-CRP MAb on the well and the anti-CRP second antibody then binds to CRP. Unbound protein and HRP conjugate are washed off by wash buffer. Upon the addition of the substrate, the intensity of colour is proportional to the concentration of CRP in the samples. A standard curve is prepared relating color intensity to the concentration of the CRP.

**Materials provided:**

1. Microwells coated with CRP MAb.
2. CRP Standard 6 vials (ready to use).
3. CRP Enzyme Conjugate1 bottle (ready to use).
4. Substrate 1 bottle (ready to use).
5. Stop solution 1 bottle (ready to use).
7. 20X Wash concentrate 1 bottle.

Procedure:

Prior to assay, allow reagents to stand at room temperature (18-25°C).

Gently mix all reagents before use.

1. Place the desired number of coated strips into the holder.

2. Dilute patient samples and controls 1:100 by adding 5 µl of samples to 495 µl of sample Diluent (Standars are ready to use).

3. Dispense 10 µL of standard, diluted samples and controls into the appropriate wells.

4. Add 100 µL of enzyme conjugate to all wells. Tap the holder to remove air bubbles from the liquid and mix well.

5. Incubate for 60 minutes at room temperature (18-26°C).

6. Remove liquid from all wells. Wash wells three times with 300 µL of 1X wash buffer. Blot on absorbent paper towels.
7. Add 100μL of substrate to all wells.

8. Incubate for 15 minutes at room temperature.

9. Add 50μL of stop solution to all wells. Shake the plate gently to mix the solution.

Read absorbance on ELISA Reader at 450nm within 15 minutes after adding the stopping solution.

**Calculation of Results:**

The standard curve is constructed as follows:

1. Check CRP standard value on each standard vial. This value might vary from lot to lot.

2. To construct the standard curve, plot the absorbance for the CRP standards (vertical axis) versus the CRP standard concentrations (horizontal axis) on a linear graph paper. Draw the best curve through the points.

3. Read the absorbance for controls and each unknown sample from the curve. Record the value for each control of unknown sample.

4. The obtained values of the patient samples and control sera should be multiplied by the dilution factor of 100 to obtain CRP results in mg/L.
5. Patient samples with CRP concentrations greater than 10mg/l should be further diluted 10-fold after the initial 100-fold dilution (total dilution 1:1,000), and the final CRP values should be multiplied by 1,000 to obtain CRP results in mg/L.
4.2.4. Estimation of plasma fibrinogen by immunochemical (Nephelometric) method

**Principle of the assay:** Estimation of fibrinogen is possible by immunochemical method. In an immunochemical reaction, the fibrinogen contained in the human plasma sample form immune complex with specific antibodies. These complexes scatter a beam of light passed through the sample. The intensity of scattered light is proportional to the concentration of fibrinogen in the sample. The result is evaluated by comparison with a standard of known concentration.

**Reagent:**

Antiserum to human fibrinogen (1x2ml): It is liquid animal sera and is produced by immunization of rabbit with highly purified human fibrinogen. Sodium azide (<1g/L) present in antisera as preservative. The antisera are ready-for-use as supplied and require no additional preparation.

**Precaution:**

Turbidity and particles in the sample may interfere with determination. Therefore, sample containing particles must be centrifuged prior to testing.

**Procedure:**

Samples are automatically diluted (1:20) with diluent. All steps are performed automatically by the system. Referance curves are constructed by
multipoint calibration. Several dilutions of standard are automatically prepared by the instrument using diluent.

4.3. Cardiac markers

4.3.1. Determination of aspartate aminotransferase (AST) by kinetic method:

**Principle of the assay:**

Aspartate aminotransferase catalyses the transfer of the amino group from aspartate to α-ketoglutarate forming oxaloacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH measured at 340 nm by means of malate dehydrogenase (MDH) coupled reaction.

\[
\text{Aspartate} + \alpha - \text{ketoglutarate} \rightarrow_{\text{AST}} \text{Oxaloacetate} + \text{Glutamate}
\]

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightarrow_{\text{Malate dehydrogenase}} \text{Malate} + \text{NAD}^+
\]

**Composition:**

**Reagent A:** Tris 121 mMol/l, L-aspartate 362 mMol/l, malate dehydrogenase > 460 U/l, Lactate dehydrogenase > 660 U/l, Sodium hydroxide 255 mMol/l, pH 7.8
**Reagent B**: NADH 1.3 mMol/l, 2 oxaloglutarate 75mMol/l, sodium hydroxide 148 mMol/l, sodium azide 9.5 g/l.

Working reagent: The contents of the reagent B was added into the reagent A bottle (4 ml Reagent A: 1 ml Reagent B), mixed well and stored at 2 – 8 °C.

**Procedure**:

One ml working reagent was taken in the cuvette and 100 μl of serum sample was added. It was mixed well and incubated for 30 seconds. After noting the initial absorbance, absorbances were recorded every one minute for three minutes. The difference between the absorbances and the average absorbance per minute (A/min) was calculated

4.3.2. **Determination of creatine kinase-MB (CK-MB) by Immunoinhibition method**

**Principle of the assay**:

Immunoinhibition method whereby specific antibodies inhibit the activity of the CK-M subunit without affecting the activity of the CK-B subunit. Because the CK-BB activity in the circulation is negligible, the activity measured by this method and multiplied with a factor of 2 reflects the activity of CK-MB.
Creatinine phosphate + ADP \rightarrow Creatine + ATP

\text{HK}

Glucose + ATP \rightarrow Glucose-6-phosphate + ADP

\text{G6P-DH}

Glucose-6-P + NADP \rightarrow Gluconate-6-P + NADPH + H^+

\textbf{Reagents and Contents:}

\textbf{10 x 8ml Enzymes}

- Imidazole buffer (pH 6.5) 0.1mol/l
- Glucose 20mmol/l
- Magnesium acetate 10 mmol/l
- N-acetylcysteine 0.2mmol/l
- AMP 5mmol/l
- Diadenosine pentaphosphate 10\textmu mol/l
- NADP 2mmol/l
- HK >4 U/ml
- EDTA 2mmol/l
- SH-stabiliser 30mmol/l
- Anti-CK antibodies (goat) blocking 2000 U/l CK-MM
- Capacity up to

\begin{itemize}
  \item Sodium azide 0.095%
  \item 2 x 10ml substrate
\end{itemize}

\textbf{Substrate}

- ADP 2mmol/l
- G6P-DH >2.8 U/ml
- Creatine phosphate 30mmol/l
Reagent preparation:

Enzyme and substrate are ready to use for the reagent start method.

To prepare the working reagent, mix 4 parts ENZ with 1 part SUB.

Reagent stability:

Enzyme and substrate are stable up to the stated expiry date when sealed and stored at 2-8°C. After opening the reagents are stable for 30 days at 2-8°C. Contamination of the reagents must be avoided. The working reagent is stable for 30 days at 2-8°C and for 2 days at 15-25°C.

Assay:

Wavelength : 340nm

Optical path : 1cm

Temperature : 25°C, 30°C or 37°C

Measurement : Against air (increasing absorbance)

Pipetting Scheme for Sample start:

Bring working reagent to the desired temperature and keep the temperature constant (+0.5°C) for the duration of the test.
Pipetting into cuvettes 25°C, 30°C 37°C
Sample 100 l 50 l
Working reagent 1000 l 1000 l

Mix and incubate for 5 minutes at the desired temperature. Read the absorbance and at the same time start the stopwatch. Read the absorbance again exactly after 1, 2 and 3 minutes.
Calculation:

Using the absorbance readings calculate the mean absorbance change per minute. (\(\frac{\Delta A}{\text{min}}\))

Calculate the CK-MB activity in the sample by multiplying \(\frac{\Delta A}{\text{min}}\) using the factor either by 3492 or 6666 (depending on temperature selected)

<table>
<thead>
<tr>
<th>Sample start</th>
<th>wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C, 30°C</td>
<td>340nm</td>
</tr>
<tr>
<td>37°C</td>
<td>3492</td>
</tr>
<tr>
<td></td>
<td>6666</td>
</tr>
</tbody>
</table>

Conversion factor: 1U/l = 16.67x10.3\(\text{kat/l}\)

1\(\text{kat/l}\) = 60U/l

4.3.3. Determination of Troponin T by electro chemi luminescence immunoassay (ECLIA)

**Principle of the assay**: The cardiac Troponin T assay employs two monoclonal antibodies specifically directed against human cardiac troponin T. The antibodies recognize two epitopes (amino acid position 125-131 and 136-147) located in the central part of the cardiac troponin T protein, which consists of 288 amino acids.

Cardiac troponin T in serum sample, a biotinylated monoclonal anti-cardiac troponin T-specific antibody, and a monoclonal anti-cardiac troponin
T-specific antibody labeled with a ruthenium complex react to form a sandwich complex. After addition of streptavidin-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 the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve (5-point calibration) provided via the reagent barcode.

**Reagents - working solutions**

1. Streptavidin-coated microparticles, 1 bottle, 12mL; Streptavidin-coated microparticles 0.72 mg/mL; preservative.

2. Anti-troponin T-Ab-biotin, 1 bottle, 14 mL: Biotinylated monoclonal anti-cardiac troponin T-antibody (mouse) 2.5 mg/L; phosphate buffer 100 mmol/L, pH 6.0; preservative; inhibitors.

3. Anti-troponin T-Ab Ru(bpy), 1 bottle, 14 mL: Monoclonal anti-cardiac troponin T-antibody (mouse) labeled with ruthenium complex 2.5 mg/L; phosphate buffer 100 mmol/L, pH 6.0; preservative.
Bring the cooled reagents to approx. 20 °C and place on the reagent disk (20 °C) of the analyzer. Avoid the formation of foam. The system automatically regulates the temperature of the reagents and the opening/closing of the bottles.

**Calculation**

The analyzer automatically calculates the analyte concentration of each sample (either in pg/mL, ng/mL or optional in μg/L).

**4.3.4. Determination of Troponin I by immunoassay**

**Principle of the assay:** The assay principle combines a one step immunoassay sandwich method with final fluorescent detection (ELFA). The solid phase receptacle (SPR) serves as a solid phase as well as the pipetting device for the assay. Reagents for the assay are ready to use and predispensed in the sealed reagent strips.

All of the assay steps are performed automatically by the instrument. The sample is transferred into the wells containing anticardiac troponin antibodies labeled with alkaline phosphatase (conjugate). The sample/conjugate mixture is cycled in and out of the SPR several times. This operation enables the troponin I to bind with the immunoglobulins fixed to the interior wall of the SPR and the conjugate to form a sandwich. Unbound components are eliminated during washing steps.
Two detection steps are then performed successively. During each step, the substrate (4 methyl-umbeliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyses the hydrolyses of substrate into a product (4-Methyl-Umbeliferone), the fluorescence of which is measured at 450nm. The intensity of the fluorescence is proportional to the antigen present in the sample.

At the end of the assay the results are automatically calculated by the instrument in relation to the calibration curve stored in the memory corresponding to the two detection steps. A fluorescence threshold value determines the calibration curve to be used for each sample.

**Reagents**

1. 60 strips STR Ready-to-use
2. 60 SPRs SPR Ready-to-use
3. Controls C1
   Control C1 (2x2ml lyophilized)
   Control C2 (2x2ml lyophilized)
4. Calibrators S1
   Calibrator S1 2x2ml (lyophilized)
   Calibrator S2 2x2ml (lyophilized)
5. Diluents R1 Ready to use
   (1x2ml)

   Human serum + Troponin + Preservative
**SPR:** The interior of the SPR is coated during production with mouse monoclonal anticardiac troponin I immunoglobulins. Each SRP is identified by the code.

**The Strip:** The strip consist of 10 wells covered with a labeled, toil seal. The label comprises a bar code which mainly indicates this assay code, kit lot number and expiration date. The foil of the first well is perforated to facilitate the introduction of the sample. The last well of each strip is a cuvette in which the fluorometric reading is performed. This well in the centre section of the strip contain the reagent.

**Procedure:**

1. Remove the required reagents from the refrigerator.

2. Use one strip and one SPR for each sample, control and calibrator to be tested. Make sure that the storage pouch has been carefully released after the required SPRs have been removed.

3. The test is identified by the code on the instrument. The calibrators must be identified by S1 and S2 and tested in duplicate. If the control needs to be tested, they should be identified by “C1” and “C2” and tested in simple.

4. Mix the calibrator and/or controls and/or samples using a vortex-type mixture (for serum or plasma separated from pellet).
5. For this test the calibrator, control and sample test portion is 200 ul.

6. Insert the SPRs and strips into the instrument. Check to make sure the colour labels with the assay code on the SPRs and the reagent strips match.

7. Initiate the assay immediately. All the assay steps are performed automatically by the instrument.

8. Reclose the vials and return them to the required temperature after pipetting.

9. The assay will be completed within approximately 20min. After the assay is completed, remove the SPRs and strips from the instrument.

10. Dispose of the used SPRs and strips into an appropriate recipient.

**Results and Interpretation:** Once the assay is completed, results are analysed automatically by the computer. Fluorescence is measured twice in the Reagent strips, reading curette for each sample tested. The results are automatically calculated by the instrument and the concentration is expressed in g/L.

**4.4. Estimation of blood glucose by oxidase/ peroxidase method:**

**Principle of the assay:** Glucose oxidase oxidizes the specific substrate, β D glucose to gluconic acid and hydrogen peroxide is generated. Hydrogen
peroxide thus produced is acted upon by peroxidase and oxygen is liberated. The liberated oxygen is transferred to chromogen system consisting of 4-aminoantipyrine and phenolic compound to produce red quinonimine dye. The intensity of colour is directly proportional to the concentration of the solution and it is determined using an auto analyser.

\[
\text{Glucose} + \text{H}_2\text{O} \xrightarrow{\text{Glucose Oxidase}} \text{Gluconate} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Peroxidase}} \text{Nascent oxygen} + \text{H}_2\text{O}
\]

Nascent oxygen + Phenolic compound + 4 – Aminoantipyrine \longrightarrow Coloured Complex

**Reagent composition:**

Glucose reagent: It contains glucose oxidase 10 KU/l, peroxidase 1.5 KU/l, phenol 6 mMol/l, 4-Aminoantipyrine 1 mMol and acetate buffer 180 mMol.

**Procedure:**

<table>
<thead>
<tr>
<th>Contents added</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
The contents in each tube was mixed well and kept at 37 °C for ten minutes. The absorbance of the standard and test were read at 505 nm against a reagent blank.
Calculation:

4.5. Statistical analysis:

The data were analysed using the computer software, Statistical Package for social sciences (SPSS) version 17. Data were expressed in its frequency, percentage and graphs. The mean value of quantitative variables was compared in the MI patients and control group by the independent-samples t-test procedure – 2.

\[ t = \frac{\text{difference in the mean values}}{\text{SE (difference in the mean values)}} \]

Where,

SE is standard error.
For statistical evaluation, a two-tailed probability of value, $p < 0.05$ was considered significant.