MATERIALS AND METHODS

Study Design **-** A case control prospective study was conducted in department of biochemistry in a tertiary care hospital. Total 172 patients were included in this study and were divided into two groups.

Group I (Cases)- consisted of 100 Diagnosed case of non-diabetic Myocardial infarction admitted in emergency & ICUs of the Santosh Medical college Hospital &RC Ghaziabad and Combined District Hospital Sanjay Nagar Ghaziabad.

Group II (controls)- consisted of 72 Healthy subjects with no history of diabetes and/or myocardial infarction.

Informed consent was taken from patients. Information extraction forms were used to collect data from patient files.

Inclusion and exclusion criteria was followed:

Inclusion Criteria –

- All cases of non diabetic Myocardial infarction. Diagnosis will be based on ECG findings & Cardiac enzymes (Trop – T / CPK- MB)
- Sex – Both (male & female)
- Age – more than 35 years of age

Exclusion Criteria -

Patients with diabetes mellitus having myocardial infarction, recent history of surgery and trauma within the preceding 2 months, renal insufficiency (serum creatinine >1.5), Patients with cerebrovascular accidents or pervious history of cerebrovascular accidents, patients having

evidence of infections, inflammatory disease, malignancy, patient taking drugs like vitamin B-complex or folic acid, hormone replacement therapy and those who are not willing to participate.

Sample collection – The initial evaluation of patients with myocardial infarction consisted of history, physical examination, ECG changes and laboratory investigation (CKMB and Troponin T). The blood samples were collected from all the cases and controls and were immediately analysed for biochemical parameters.

Under all aseptic precautions, about 5 ml of venous blood samples was collected by clean venepuncture in a sterile plain & EDTA vial. Blood samples were allowed to coagulate after which they were centrifuged at 3000 rpm for 5minutes to obtain sera. The separated clear sera was transferred into sterile bottles and used for the enzyme assay. When not used immediately, they were stored at -20°C and later used within 5 days.

SAMPLE SIZE

Evaluation of Clinical Utility of Serum Enzymes, Lipid Profile, Homocysteine in Early Stages of Acute Myocardial Infarction was observed by Ch Sankeerthi, A Vaithialingam, T Sandhya Rani, BS Ravi Kiran, T Mohanalakshmi,and E Prabhakar Reddy.[1]

The study observed that average incidence of myocardial infarction for those aged between 30 and 69 years was about 600 per 1, 00,000 for men and 200 per 1, 00,000 for women. Also the study observed mean values of homocysteine in cases was 17.73 ± 1.52 and in control was 12.40 ± .99. Taking these values as reference, the minimum required sample size with 90% power of study, 5% level of significance and 1.5% margin of error is 102 patients. Taking a minimum ratio of 1:1 for cases and controls, minimum of 51 cases and 51 controls were included in the study.

<u>Formula used is:-</u>

1) For comparing mean of two groups

$$N>=2(\text{standard deviation})^2 *(Z_\alpha + Z_\beta)^2 /(\text{mean difference})^2$$

Where Z_α is value of Z at two sided alpha error of 5% and Z_β is value of Z at power of 90% and mean difference is difference in mean values of two groups.

2) $N \geq ((i(1 - i))/(ME/z_\alpha)^2$

Where Z_α is value of Z at two sided alpha error of 5%, ME is margin of error and i is incidence rate.

Calculations:-

1) N>=((.006*(1-.006)/(.015/1.96)^2=101.828=102(approx.).
2) N>=((.002*(1-.002)/(.015/1.96)^2=34.079=35(approx.).
3) Pooled standard deviation=sqrt((1.52*1.52+.99*.99)/2)=1.28

N>=(2(1.28*1.28)*(1.96+1.28)^2)/(17.73-12.40)=1.21=2(approx.)

<u>Measurement of Troponin T</u> –

Measurement of Troponin T will be done qualitatively by rapid test kit method manufacturing by Roche Diagnostics International Ltd CH-6343 Rotkreuz, Switzerland

Assay Procedure –

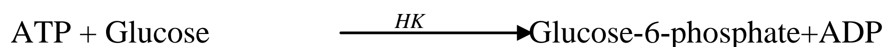
- Obtain the blood sample. Remove strip from package and place on a flat surface.
- Draw 150 µL of blood into dispensing pipette.
- Apply sample on the test strip.
- Read results after 15 minutes. 1 line (control line) = negative test result (troponin T concentration < 100 ng/L)
- Two lines (control and signal line) = positive test result (troponin T concentration > 100 ng/L)

Measurement Of Creatinine Kinase (CK-MB)

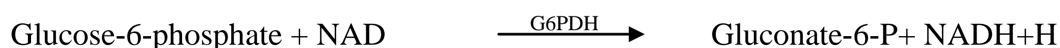
Measurement of CK-MB will be done following a method that has been made according to the International Federation of Clinical Chemistry (IFCC) protocol. Commercial kits produced by Transasia Biomedical Ltd. (H.P.) are used. The test was conducted using the mono-reagent procedure[36].

Principle Specific antibodies against CK-M inhibit the complete CKMM activity and the CK-M subunit of CKMB. Only CK-B activity is measured.

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CKBB/CKMB}} \text{Creatine} + \text{ATP}$$



$$\text{ATP} + \text{Glucose} \xrightarrow{HK} \text{Glucose-6-phosphate+ADP}$$



$$\text{Glucose-6-phosphate} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{Gluconate-6-P+ NADH+H}$$

REAGENT COMPOSITION (R1)	
Imidazole buffer, pH 6.1	125 mmol/l
Glucose	25 mmol/l
Magnesium acetate	12.5 mmol/l
EDTA	2 mmol/l
N-acetyl-L-cysteine	25 mmol/l
NADP	2.4 mmol/l
Hexokinase	> 6.8 U/ml Anti-CK antibodies (goat) blocking capacity up to 2000 U/l CK-MM

REAGENT COMPOSITION (R2)	
ADP	15.2 mmol/l
D-glukoso-6-phosphate-dehydrogenase	> 8.8 U/ml
Creatine phosphate	250 mmol/l
AMP	25 mmol/l
Diadenosinepentaphosphate	103 µmol/l

Reagent Prepration –

Reagents (R1) & Reagents (R2) are liquid, ready to use.

Stability and storage of Reagents

The unopened reagents are stable till the expiry date stated on the bottle and kit label when stored at 2–8°C. After the first opening the vials, reagents are stable for 30 days at 2–8°C in the dark.

Assay Procedure

Two reagents method – substrate

Reagent 1 (Buffer)	1.000 ml
Sample	0.050 ml

Mix and incubate for 3 min. at 37°C. Then add:

Reagent 2 (Substrate)	0.250 ml

Mix and incubate for 3 min. at 37 °C. Then measure the absorbance and at the same time start the stopwatch. Read the absorbance again exactly after 1, 2 and 3 minutes. Calculate the average 1 minute absorbance change (ΔA).

Calculation –

CK (U/l) = $\frac{\Delta A\ sam1.}{\Delta A\ cal}$x Ccal   Ccal = calibrator concentration

Using factor: CK (U/l) = f x ΔA/min

f = factor

f = 4127 (at 340 nm)

<u>Measurement of C – Reactive Protein (CRP) -</u>

Measurement of CRP was done following a method that has been made according to the measurement of antigen antibody reaction by the end point method. Commercial kits produced by ErbaLachemas.r.o., Karasek 1d, 621 00 Brno, CZ are used[34]

Reagent Provided

Buffer	
Phosphate buffered saline	pH 7.43
Polyethylene glycol	40 g/l
Sodium azide	0.09 %

Antiserum	
Phosphate buffered saline	(pH 7.43)

Polyclonal goat anti-human CRP (variable). Sodium azide	(0.09 %)

Calibrator

Pooled human serum,diluted with high levels of CRP with phosphate buffered saline, liquid and stabilized. Contains 0.09 % sodium azide as preservative.

Reagents required but not supplied

Saline (9 g/l NaCl)

Manual Procedure

Sample/Control dilution: None Reference curve: Generate a reference curve by successive 1:2 dilutions of Calibrator High in saline or use the ready for use calibrator set. Use saline as zero point.

Test: Mix 60 µl of calibrators and samples with 1000 µl of buffer. Read optical density (OD1) of calibrators, controls and samples at 340 nm. Add 100 µl of CRP antiserum. Mix and incubate for 5 minutes at room temperature. Read optical density (OD2) of calibrators, controls and samples at 340 nm. Calculate ΔOD's, plot a calibrator curve and read the concentration of controls and samples..

Reference Values

0 – 10 mg/l (IFCC),resp. 0-1 mg/dl

This range is given for orientation only. Each laboratory should establish its own reference values.

Measurement of Lactate Dehydrogenase (LDH)

Measurement of Lactate Dehydrogenase (LDH) was done following a method that has been made according to the International Federation of Clinical Chemistry (IFCC) protocol. Commercial kits produced by Transasia Biomedical Ltd. (H.P.) are used. The test was conducted using the monoreagent procedure.[37]

Principle

The LDH method is based on the recommendations of DGKCH (from pyruvate). This reagent uses pyruvate and is based on the method of Henry et al.

Pyruvate + NADH ⟶ Lactate + NAD+

LDH catalyses the reduction of pyruvate to lactate oxidising reduced nicotinamide adenine dinucleotide (NADH) to NAD. The activity of LDH can be determined by the rate of decrease in absorbance at 340 nm as NAD is produced.

Reagent Composition –

Reagent 1 (R1)	
Tris Buffer (pH 7.5)	100 mmol/l
Pyruvat	2.0 mmol/l

Reagent 2 (R2)	
NADH	1.66 mmol/l

Reagent Prepration –

Reagents (R1) & Reagents (R2) are liquid, ready to use.

Assay Procedure –

	Reagent blank	Calibrator	Sample
Reagent 1	0.800 ml	0.800 ml	0.800 ml
Sample	-	-	0.020 ml
Calibrator	-	0.020 ml	-
Distilled water	0.020 ml	-	-

Mix and after 1 min. incubation (at 37°C) add

Reagent 2	0.200 ml	0.200 ml	0.200 ml

Mix, incubate 1 min. at 37°C and then measure the initial absorbance of calibrator and sample against reagent blank. Measure the absorbance change exactly after 1, 2 and 3 min. Calculate 1 minute absorbance change (ΔA/min).

Calculation –

LDH (U/l) = $\frac{\Delta Asam/min}{\Delta Acal/min}$ x Ccal   Ccal = calibrator concentration

Using factor: LDH (U/l) = f x ΔA/min

f = factor

f = 8095 (at 340 nm)

Measurement of Serum Homocysteine level

Serum homocysteine level was estimated in serum sample using Enzyme Linked Immunosorbent Assay (ELISA). [119,120] Elisa kit manufactured by Qayee-Bio Ltd. China and Elisa reader of Robonik (india) pvt.ltd. were used in this study.

Kit Components

Box 1 (shipped at room temperature)

1. 96 Well Protein Binding Plate: One strip well 96 well plate.
2. Anti-Homocysteine Antibody (500X): One 15 µL vial.
3. Secondary Antibody, HRP Conjugate (1000X): One 20 µL vial.
4. Assay Diluent: One 50 mL bottle.
5. 10X Wash Buffer: One 100 mL bottle.
6. Substrate Solution: One 12 mL amber bottle.
7. Stop Solution: One 12 mL bottle.

Box 2 (shipped on blue ice packs)

1. Homocysteine Conjugate (1000X) : One 20 µL vial.
2. Homocysteine-BSA Standard: One 20 µL vial of 4 mg/mL homocysteine conjugated to BSA in PBS.

Storage

Upon receipt, store Homocysteine Conjugate (1000X) and Homocysteine-BSA Standard at -20ºC. Store the rest of the kit at 4ºC. Storage

Preparation of Reagents

Homocysteine Conjugate Coated Plate: Determine the number of wells to be used, and dilute the Homocysteine Conjugate 1:1000 into PBS. Add 100 µL of 1X homocysteine conjugate to each well of the 96-well Protein Binding Plate. Incubate for 2 hrs at 37ºC or overnight at 4ºC. Remove the diluted homocysteine conjugate, blotting plate on paper towels to remove excess fluid. Wash wells 3 times with 200 µL of PBS and blot on paper towels to remove excess fluid. Add 200 µL of Assay Diluent to each well and block for 1 hour at room temperature. Transfer the plate to 4ºC until ready to begin the assay.

Note: The Homocysteine Conjugate Coated Plate is not stable long-term. We recommend using it within 24 hours after coating.

• 1X Wash Buffer: Dilute the 10X Wash Buffer to 1X with deionized water. Stir to homogeneity.

• Anti-Homocysteine Antibody and Secondary Antibody, HRP Conjugate: Immediately before use dilute the Anti-Homocysteine Antibody 1:500 and the Secondary Antibody, HRP Conjugate 1:1000 with Assay Diluent. Do not store diluted solutions.

Prepration of Sample

The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

- Plasma: Collect blood with heparin or EDTA and centrifuge for 10 minutes at 1000 g at 4ºC. Remove the plasma and assay immediately or store samples at -80ºC for up to three months. Normal plasma samples should be diluted 2- to 10-fold with PBS containing 0.1% BSA immediately before running the ELISA.

- Serum: Harvest serum and centrifuge for 10 minutes at 1000 g at 4ºC. Assay immediately or store samples at -80ºC for up to three months. Normal serum samples should be diluted 2- to 10-fold with PBS containing 0.1% BSA immediately before running the ELISA.

- Tissue homogenate: Weigh and homogenize the tissue on ice in 5-10 mL cold PBS per gram of tissue. Centrifuge at 10,000 x g for 15 minutes at 4°C. Remove the supernatant and store on ice. Store any unused supernatant at -80°C for up to three months.

- Cell lysate: Collect cells by centrifuging at 2000 x g for 10 minutes at 4°C. Sonicate or homogenize the cell pellet on ice in 1-2 mL cold PBS. Centrifuge at 10,000 x g for 15 minutes at 4°C. Remove the supernatant and store on ice. Aliquot and store the

supernatant for use in the assay. Store any unused supernatant at -80°C for up to three months.

- Other biological fluids: Centrifuge samples for 10 minutes at 1000 g at 4ºC and recover supernatant. Assay immediately or store samples at -80ºC for up to three months.

Assay Procedure

1. Prepare and mix all reagents thoroughly before use. Assay Protocol
2. Each unknown sample (see Preparation of Samples section), Homocysteine-BSA standard, and blank should be assayed in duplicate.
3. Remove the Assay Diluent from the plate and add 50 µL of unknown sample or standard to the Homocysteine Conjugate Coated Plate. Incubate at room temperature for 10 minutes on an orbital shaker.
4. Add 50 µL of diluted Anti-Homocysteine Antibody (see Preparation of Reagents section) to each well. Incubate at room temperature for 1 hour on an orbital shaker.
5. Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After each wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
6. Add 100 µL of the diluted Secondary Antibody, HRP Conjugate to each well. Incubate at room temperature for 1 hour on an orbital shaker. During this incubation, warm Substrate Solution to room temperature.

7. Wash the strip wells 3 times according to step 5 above. Proceed immediately to the next step.

8. Add 100 μL of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes.

Note Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.

9. Stop the enzyme reaction by adding 100 μL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).

10. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

ECG

Patient was requested to lie down on a comfortable bedding. Privacy was maintained. The chest was exposed. ECG was done by using the portable ECG machine. 4 long leads were attached on both legs and both wrists and 6 short leads were placed on chest (1 on each side of sternum at the level of nipple near the costochondral junction, third was placed between nipple and sternum at 5th intercostal space, 1 was placed below nipple in 6th intercostal space, 1 was placed between nipple and mid-axillary line in 5th intercostal space, and the last one at mid axillary line at the level of nipple) with the help of the gel and the vaccum in the lead pump.