CHAPTER III  MATERIALS AND METHODS

3.1. Collection of subject’s data

The total number of participants included in this study was three hundred (300), all registered for a medical check-up at Vinayaka Mission’s Medical College and Hospital, Karikal, between April 2011 to June 2013. At the time of admission or entrance all patients responded to a standardized questionnaire covering many personal details (such as smoking history, alcohol intake, physical activity, food pattern, family history of CHD, and medical information with the prescribed drugs) organised by trained interviewers. The data regarding the patients were scheduled. From that assessment the data was used for further analysis.

3.2. Characterization of the study subjects

Smoking status was divided into never smoker and ever smoker, and alcohol status in to never drinker and ever drinker. The term “never” means the person who never smokes nor drinks in his or her lifetime and “ever” was used to classify the person who had smoked or drunk for at least one month or currently smokes or drinks.

Hypertension was defined as systolic blood pressure (SBP) = 130 mmHg and/or diastolic blood pressure (DBP) = 90 mmHg (Chobanian et al., 2003). Obesity was commonly defined as a BMI (BMI = weight divided by height squared) of 30 kg/m² or higher (World Health Organization, 2000). Diabetes mellitus was defined as the patients who were being told by a healthcare professional that they had diabetes mellitus, taking insulin or hypoglycemic medications, had a random blood glucose level >200 mg/dL, or had a fasting (≥8 hours before venipuncture) blood glucose level ≥126 mg/dL (American Diabetes Association, 2006).

According to NCEP ATP III standard guidelines, hypercholesterolemia and hypertriglyceridemia were defined as TC and TG levels of >200 mg/dL and >150 mg/dL, respectively (Expert Panel on Detection, Evaluation, and Treatment of High
Blood Cholesterol in Adults, 2001). Low-HDL cholesterolemia was defined as HDL cholesterol level of <40 mg/dL. LDL hypercholesterolemia was defined as >100 mg/dL.

Atherogenic dyslipidemia was defined as having hypercholesterolemia, hypertriglyceridemia, and/or low-HDL cholesterolemia. According to the NCEP criteria, an individual may be diagnosed to have metabolic syndrome if he or she has three or more of the following: obesity, hypertriglyceridemia, low-HDL cholesterolemia, hypertension and diabetes (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001).

3.3. Routine laboratory measurement

All anthropometric assessment, physical assessment and blood sampling were conducted in a temperature controlled room maintained at 25±2°C.

3.3.1. Physical examinations

The SBP, DBP, Electrocardiogram (ECG), and Echocardiogram (ECHO) during the in-hospital phase of the study were measured by well-trained staff members. BP was measured in the right arm of a seated subject, after atleast 5 min of rest, with a mercury sphygmomanometer with the optimal cuff-size for the patient’s arm circumferences. To determine the SBP and DBP the first and fifth Korokoff sounds were recorded.

3.3.2. Biochemical examinations

Blood samples were obtained from the anticubital vein after overnight fasting. Blood was analysed for hsCRP, fibrinogen, TNF-α, IL-6, Lp(a), apolipoproteins (Apo AI and Apo B), glucose (fasting and post prandial), HbA1C, lipids (TC, TG, HDL cholesterol, LDL cholesterol and VLDL cholesterol) and uric acid. Liver markers (aspartate amino transferase - AST, alanine amino transferase - ALT, bilirubin, total protein, albumin, globulin and A/G ratio), kidney function (urea and creatinine), muscle (creatinine phosphokinase - CPK), creatine kinase-myocardial band isoenzyme (CK-MB), troponin T, complete blood count with differential count, hemoglobin and a
routine urine analysis were obtained in some of patients. The above specified parameters are based upon the need of the patients, which is directly under the guidance of the doctors.

3.4. Study design

Fig. 1, showed that the study design of the present study. Patients received atorvastatin, aspirin, oral or intravenous nitrates, angiotensin converting enzyme inhibitor, and beta-blockers as required according to the physician’s judgment.

3.4.1. Screening visit

At screening visit, various biochemical parameters were measured and recorded. From the recorded data, patient’s history was drawn and based on the following inclusion and exclusion criteria, patients were scrutinized and subjected to further evaluation. All patients were instructed for the Therapeutic Lifestyle Changes diet recommended by the NCEP (National Cholesterol Education Program, 1994).

3.4.1.1. Inclusion criteria

Inclusion criteria were based on the typical chest pain, changes in ECG, ECHO, initial screening with changes in biochemical parameters. Moreover the patients of the treated group received atorvastatin therapy and untreated group did not received atorvastatin therapy.

3.4.1.2. Exclusion criteria

Exclusion criteria were a previous MI within 6 months, recent major surgery, elevated body temperature (>38.0°C), renal failure and significant hepatic disease.

3.4.2. Randomization

After giving written informed consent, eligible patients were allocated to Group I (Control), Group II (Test group – untreated, patients with CVD) and Group III (Test group – treated with atorvastatin 10mg/day, patients with CVD). After having randomized the patients, the blood samples were being collected from the lab for other
biochemical estimations. The number of participants included in group I, group II and group III was forty five (45), fifty one (51) and forty two (42) respectively. Medical check-ups were scheduled at three, six and twelve (at the end of the study) months after randomization.

3.4.3. Follow-up visit

It has been carried out in the third month after the randomization and the following parameters were scheduled during the follow up visit. HsCRP, fibrinogen, TNF-α, IL-6, Lp(a), apolipoproteins, glucose, HbA1C, TC, TG, HDL cholesterol, LDL cholesterol, VLDL cholesterol, AST, ALT, bilirubin, total protein, albumin, globulin, uric acid, urea, creatinine, CPK and CK-MB were analyzed.

3.4.4. Safety visit

During safety visit at 6th month, the patients had not been taken to any biochemical analysis and patients were consulted with the doctor as a normal procedure. In the event of the absentia of the patients, they were taken to consultation procedure over phone.

3.4.5. End of the study visit

The end of the study visit was being processed after twelve month. Specifically the condition and the progress in the health of the patients were noted. End of the study is continued with the same subjects where the number is slightly reduced. Since the patients are not able to continue their regular visit. For check up due to their personnel constrains, the number has been decreased.

Total of thirty five patients were discontinued from the present study. During this visit all the physical and biochemical examinations were done similar to that of follow-up visit. The percentage of reduction as well as increase has been calculated based on the number of subject in the second visit. At the termination of this study, the patients were entirely left with the care and advice of the doctors.
3.5. List of parameters analysed in this study

The following Table 1 shows the list of parameters analysed in patients with or without CVD.

3.6. Biochemical analyses

The venous blood samples were drawn into pyrogen-free blood collection tubes without additive. The serum was collected following centrifugation at 3500 rpm for 3 minutes and then stored at 2 to 8°C for seven days and thereafter at -70°C until analyzed and stored samples were collected for further biochemical analysis. At each visit only lipid profile (TC, HDL cholesterol and TG) were estimated for the patients as per the prescription of the doctor. Blood was further analyzed for other biochemical parameters.

The concentration of hsCRP was measured in serum by the latex-enhanced immunoturbidimetric method. The method of Clauss measures the rate of fibrinogen to fibrin conversion in the presence of excess thrombin and has been shown to be rapid, sensitive and precise. The concentration of TNF-α, IL-6 and Lp(a) was measured in serum and plasma by an in vitro enzyme-linked immunosorbent assay. Apo A-I and apoB were measured by immunoturbidimetry using the auto analyser.

TC and TG were assayed by routine enzymatic methods using an auto analyser. HDL cholesterol was estimated using the same enzymatic method after precipitation of the plasma with phosphotungstic acid in the presence of magnesium ions. Majority of testing methods for LDL cholesterol estimation do not really measure LDL cholesterol in their blood, much less particle size. Because of cost, LDL cholesterol values have long been estimated using the Friedewald formula: [TC] – [total HDL cholesterol] – 20% of the TG value = estimated LDL cholesterol.

The basis of this is that TC is defined as the sum of HDL cholesterol, LDL cholesterol, and VLDL cholesterol. Normally just the total, HDL cholesterol, and TG are actually measured. The VLDL cholesterol is estimated as one-fifth of the TG. It is important to fast for at least eight hours before the blood test because the TG level varies
significantly with food intake. Non-HDL cholesterol value has calculated as TC - HDL cholesterol.

For photometric determination of the glucose (GOD POD method), HbA1C (immunoassay), uric acid (Uricase method), total bilirubin (Jendrassik and Grof method), AST, ALT, total protein and albumin based on reference method of International federation of Clinical Chemistry, urea (“Urease – GLDH”: enzymatic UV test), creatinine (Jaffe kinetic method). CPK was determined colorimetrically. CK-MB was determined by in vitro method. The following Table 2 shows the sources of kits with references.

The various ratios were calculated as follows:

**Ratios**

TC to HDL cholesterol = TC/HDL cholesterol

LDL cholesterol to HDL cholesterol = LDL cholesterol/HDL cholesterol

TG to HDL cholesterol = TG/HDL cholesterol

Non-HDL cholesterol to HDL cholesterol = non-HDL cholesterol/HDL cholesterol

Apo B to apo A-I = apo B/apo A-I

Apo B to HDL cholesterol = apo B/HDL cholesterol

Albumin to Globulin (A/G) ratio = A/G

**Percentage of reduction and increase**

\[
\% \text{of reduction and increase} = \frac{\text{Mean value before treatment} - \text{Mean value after treatment}}{\text{Mean value before treatment}} \times 100
\]

**3.7. Statistical analysis**

Statistical analysis was performed with SPSS 14 statistical software package. Information were entered on a pre-designed proforma and managed on spreadsheet. Entries were checked for any error. Descriptive statistics used for quantitative variables
were computed by mean and standard deviation. The baseline and post-treatment hsCRP, fibrinogen, Lp(a), BNF, IL, lipid components and other biochemical parameters were averaged for each of the study groups at each visit and means compared using a paired „t‟-test to calculate the significance of changes in biochemical parameters caused by atorvastatin treatment. In this study, p<0.05 has been considered as statistically significant. The RR is the ratio of the proportions of cases having a positive outcome in two groups. RR was calculated by using MedCalc easy-to-use statistical software between group III and group I.
ENROLLMENT

SCREENING VISIT n = 300
(Cardiovascular disease)

INCLUSION CRITERIA – Reasons
- Chest pain
- Changes in ECG, ECHO
- Initial screening with hsCRP, fibrinogen, lipids etc.,

EXCLUDED

REFUSED TO PARTICIPATE

RANDOMIZED n = 138

GROUP III n = 42
(ATORVASTATIN)

GROUP II n = 51
(UNTREATED)

FOLLOWUP VISIT

SAFETY VISIT AND LOST TO FOLLOW UP - Reasons
- Patient decision n = 2
- Not taking medicine n = 1

END OF STUDY VISIT ANALYZED n = 39

FOLLOWUP VISIT

SAFETY VISIT AND LOST TO FOLLOW UP - Reason
- Patient decision n = 8

END OF STUDY VISIT ANALYZED n = 43

NOT MEETING INCLUSION CRITERIA - Reasons
- Previous MI
- Recent major surgery
- Body temperature >38.0°C
- Renal failure
- Hepatic disease

ANALYSIS

Fig. 1 Study design
<table>
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<tr>
<th>Table 2 List of parameters in patients with cardiovascular disease</th>
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<tr>
<td><strong>Anthropometrical measurements</strong></td>
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<td><strong>Blood pressure</strong></td>
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<td><strong>Physical examinations</strong></td>
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<tr>
<td><strong>Biochemical parameters</strong></td>
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<td></td>
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### Table 1 Source of Kits

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Manufactured By</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HsCRP</td>
<td>The Quality System of Diagnostic Products, USA.</td>
<td>Tietz (1995)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Tcoag Ireland Limited, Ireland</td>
<td>Day et al. (2001)</td>
</tr>
<tr>
<td>TNF</td>
<td>RayBiotech, Inc., USA</td>
<td>Bonavida (1991)</td>
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<tr>
<td>IL-6</td>
<td>Pierce Biotechnology, USA</td>
<td>Chan and Perlstein (1987)</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>Trinity Biotech, USA</td>
<td>Utermann (1989)</td>
</tr>
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<td>Apo B and Apo A-1</td>
<td>Spinreact, Spain</td>
<td>Tietz (1983)</td>
</tr>
<tr>
<td>TG</td>
<td>Reagents Applications, Ind. San Diego</td>
<td>Trinder (1969)</td>
</tr>
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<td>HDL cholesterol, LDL cholesterol and VLDL cholesterol</td>
<td>Nicholas Piramal India Ltd., Navi Mumbai</td>
<td>Friedewald et al. (1972)</td>
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<td>HbA1C</td>
<td>Beckman Coulter, Ireland</td>
<td>Goldstein et al. (2004)</td>
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<td>Urea</td>
<td>DDS Diagnostik Sistemler Istanbul, Turkey</td>
<td>Thomas (1998)</td>
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<tr>
<td>Creatinine</td>
<td>Raichem, Division of Hemagen Diagnostics, Inc. San Diego</td>
<td>Tietz (1987)</td>
</tr>
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<td>CPK</td>
<td>BioAssay Systems, USA</td>
<td>Bishop et al. (1971)</td>
</tr>
<tr>
<td>CK-MB</td>
<td>Adaltis, Italy</td>
<td>Peace and Kaplan (1987)</td>
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</tbody>
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