Materials & Methods
Study Design: Cross-Sectional study and observational follow-up of the cohort

Study Setting: Department of Cardiology, Kasturba Medical College Hospital, Ambedkar Circle, Mangalore.

Sample Size: The sample size was found to be 290. It was calculated using G*Power software (Version: 3.1.2) with anticipated effect size 0.1, power 90%, level of significance 5%, number of predictors 16.

Duration of the Study: 36 months starting from November 2013.

In this study 290 type 2 diabetic patients who underwent coronary angiogram for the evaluation of clinically suspected coronary artery disease were recruited. A detailed history was taken from all subjects regarding duration of diabetes, familial status of diabetes, treatment for type 2 diabetes mellitus, smoking and alcohol status, dyslipidemia, hypertension and familial history of coronary artery disease.

A written informed consent was obtained from all the study participants after explaining the details and risks involved in the study. The study protocol was approved by our institutional ethics committee.

Inclusion Criteria: Patients with Type 2 Diabetes Mellitus fulfilling the diagnostic criteria as recommended by the American Diabetes Association (ADA) (18), and who underwent coronary angiogram for the evaluation of clinically suspected coronary artery disease were included in the study. The subjects were referred for coronary angiogram only when they were found to have significant changes in electro cardio gram (ECG) or mild positive tread mill test (TMT) as determined by a cardiologist.

The age of the study population was restricted to 45 to 65 years of age, because beyond 65 years the degree of severity of coronary artery disease is found to be same in all population (76, 357).

Exclusion Criteria: Patients with inflammatory diseases, chronic renal and liver complications, on steroid supplement and those who are on exogenous insulin administration were excluded from the study. For measurement of insulin resistance the glucose and insulin concentrations are needed to be in a steady state, thus is not recommended to measure insulin resistance following an exogenous insulin administration (137).
Measurements:

A detailed history was taken from all study participants regarding the duration of diabetes, familial status of diabetes, treatment for type 2 diabetes mellitus, smoking and alcohol status and familial history of coronary artery disease. Non-smokers were defined as those, who have abstained from smoking from past two years.

Blood Collection

Samples of blood were collected by venipuncture technique following a consent. The blood was collected two weeks after coronary angiogram in order to avoid the acute stress that is caused during the procedure, and due to the burden of the disease which could cause the fluctuations in the biochemical parameters (358). The collection, handling and storage of blood samples were considered a vital component in order to minimize any inter-sample differences.

For glucose estimation, the blood was collected in fluoride containing vacutainers. The blood was then centrifuged at 3500 rpm for 10 minutes to separate red blood cells from plasma. The separated plasma was then used for the estimation of fasting glucose.

For other biochemical parameters, the blood was collected in plain vacutainers (without anticoagulant), it was then allowed to clot for 30 min. The blood was then centrifuged at 3500 rpm for 10 minutes. The top yellow layer of serum was separated out and used for estimation. The serum was stored at -20°C for further use.

Following biochemical and clinical assays was carried out.

1) Anthropometric parameters of obesity, as per WHO recommendations
2) Systolic Blood Pressure
3) Fasting lipid profile
4) Fasting Glucose
5) Fasting Insulin
6) HbA1c
7) Microalbumin
8) Ejection Fraction
9) Severity of coronary artery disease
1) **Anthropometric Parameters:** As per WHO norms (359)

**Height:** It was measured against a vertical board with an attached metric rule and a horizontal head board was brought in contact with uppermost point on the head. It was recorded bare foot, with person standing on flat surface positioned so that the line of vision is straight to the body. The arms were hanging freely by the sides, and the head back and heels were in contact with the vertical board. The individual was asked to inhale deeply and maintained a full erect position. Top most point on the head with sufficient pressure to compress the hairs was taken as height to the nearest of 0.1 cm.

**Weight:** Weight was recorded without foot wear with light clothes worn on body, standing straight on the center of weighing machine with body evenly distributed between the foot by the ISI certified weighing machine to the nearest of 100gms.

**Body Mass Index (BMI):**

It is calculated as Weight (kg)/Height^2 (Mt)

**Normal Values:** 18-23 kg/m^2 (For Asian Indian population) (360).

**Waist Circumference:** It was measured in cm with a flexible measuring tape, midway between the inferior margin of the last rib and crest of the ileum, in the horizontal plane, at the end of expiration, to the nearest of 0.1 cm. The tape fit snugly and did not compress the underlying soft tissue.

**Normal Values:**

Men: 90 cm   Women: 80 cm (For Asian Indian population) (360).

**Hip Circumference:** It was also measured in cm with a flexible measuring tape at the level of maximum extension of greater trochanter bilaterally in the horizontal plane with the subject standing with arms at the sides and feet together with light clothes over the body.

**Waist-Hip ratio:**

Waist Circumference/ Hip Circumference

**Normal Values:** Men: 0.87-0.91, Women: 0.80-0.84.
**Blood Pressure:** Blood pressure is measured by using well-calibrated and maintained mercury sphygmomanometer. Prior to blood pressure measurement, each patient was requested to rest for 5 minutes in the supine or sitting position. The arm was supported and positioned at heart level and any tight cloth were removed. Appropriate cuff size was selected. The cuff was inflated over the brachial artery until disappearance of the pulse. This was followed by deflation of the cuff until the pulse re-appeared and this was recorded as estimated SBP. The cuff was re-inflated 30 mm Hg above the estimated SBP and a stethoscope was placed. After that, cuff was deflated at the rate of 2 mm Hg per second until the appearance of a rhythmic sound (SBP). Deflation was continued until disappearance of the sound (DBP). The blood pressure was measured twice and the mean was recorded. If the second measurement was significantly lower, a third reading was obtained and the mean of the last two readings was used.

**Normal value:** 140/90 mmHg.

**Fasting Lipid Profile:** Venous blood was collected in a vaccutainer without any anticoagulant. The serum was separated and analyzed using Hitachi P 800 auto analyzer. The coefficient variation is <2% and <5% for inter and intra batch respectively. Lipid profile included following six parameters

1) **Total Cholesterol (TC):** The estimation of Total cholesterol is based on Cholesterol oxidase and peroxidase (CHOD-PAP) method. The total serum cholesterol is enzymatically determined according to following reaction.

\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{4 Cholesten-3-one} + \text{H}_2\text{O}_2
\]
\[
2\text{H}_2\text{O}_2 + 4 \text{Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Red Quinone} + 4\text{H}_2\text{O}_2
\]

**Reference Value:** Up to 200 mg/dl
2) **High Density Lipoprotein Cholesterol (HDL-C):** This estimation is based on second generation enzymatic colorimetric method. The cholesterol concentration of HDL-C is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (Approximately 40%)

\[
\text{HDL-C Esters + H}_2\text{O} \xrightarrow{\text{PEG Cholesterol Esterase}} \text{Cholesterol + R-COOH}
\]

\[
\text{HDL-C Esters + O}_2 \xrightarrow{\text{PEG Cholesterol Oxidase}} \Delta^\frac{1}{2} \text{- Cholesteneone + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4 \text{ Aminoantipyrine + HSDA + H}^\text{+} + \text{H}_2\text{O} \xrightarrow{\text{Peroxidase}} \text{Purple pigment + 5H}_2\text{O}
\]

HSDA- Sodium N-(2-hydroxy-3-sulfopropyl)-3, 5-diethoxyaniline

In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-Aminoantipyrine and HSDA to form a purple blue dye. The colour intensity of this dye is directly proportional to the cholesterol concentration.

**Reference Value:** 40-60 mg/dl

3) **Triglycerides (TG):** The estimation of triglycerides is based on GPO-PAP method. Enzymatic determination of triglycerides is based on the following reactions.

\[
\text{TG + H}_2\text{O} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol + R-COOH}
\]

\[
\text{Glycerol + ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-3-Phosphate + ADP}
\]

\[
\text{Glycerol-3-Phosphate + O}_2 \xrightarrow{\text{Glycerol-3-Phosphate oxidase}} \text{DHAP + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4 \text{ Aminoantipyrine + p-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Red quinoneimine}
\]

**Reference Value:** Up to 150mg/dl

4) **Low Density Lipoprotein Cholesterol (LDL-C):** It is calculated using the following formula

\[
\text{LDL-C} = [\text{Total Cholesterol} - (\text{HDL-C} + \text{VLDL-C})]
\]

**Reference Value:** Up to 100mg/dl

5) **Very Low Density Lipoprotein Cholesterol (VLDL-C):** It is calculated using the following formula

\[
\text{VLDL-C} = \text{Serum Triglycerides/5 mg/dl}
\]

**Reference Value:** Up to 40 mg/dl
6) **Ratio of Total cholesterol to High Density Lipoprotein-Cholesterol (HDL-C) ratio** :
   Total Cholesterol/ HDL-C

   **Reference Value:** 3.5-5

**Fasting Glucose:** Venous blood is drawn in vacutainer with anticoagulant (fluoride). The plasma was separated and analyzed using Hitachi P800 auto analyzer. The coefficient of variation is <2%.

The estimation of glucose is based on hexokinase method. Enzymatic determination of glucose is based on the following reactions.

\[
\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP} \\
\text{Glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6PD}} \text{6-Phosphogluconate} + \text{NADH} + \text{H}^+
\]

**Reference Value:** 70-100 mg/dl

**Fasting Insulin:** Venous blood was drawn in a vacutainer without anticoagulant for serum estimation of insulin. The separated serum was analyzed by Enzyme-linked immune sorbent assay (ELISA) based on sandwich principle using Insulin ELISA kit manufactured by DRG Legal, Germany. The coefficient of variation is <3%.

**Principle:** The micro titer well is coated with a monoclonal antibody, which has affinity to bind towards an antigenic site present on the insulin molecule. An aliquot of patient’s serum sample containing endogenous insulin was nested in the well coated monoclonal antibody. A biotinylated anti-insulin antibody is added to well, after an incubation for a brief period the unbound conjugate is washed off by washing process. In the second incubation step streptavidin peroxidase enzyme complex binds to the biotinylated anti-insulin antibody. The magnitude of bound complex is proportional to the concentration of insulin in the sample. The colour developed is directly proportional to the concentration of insulin present in the patient sample.

Samples was estimated in batches. The serum/plasma separated was stored at -20º.

**Reference Value:** 5-25 µIU/ml.
Glycated Haemoglobin (HbA1c): HbA1c is the product of the glycosylation of glucose with the N-terminal residue of the β-chain of haemoglobin. The HbA1c concentration in blood is directly proportional to the mean concentration of glucose prevailing in the previous 6-8 weeks, equivalent to the life time of the erythrocytes. HbA1c was analyzed by High performance liquid chromatography (HPLC).

Reference Value: 3.0-6.5

Microalbuminuria

Microalbuminuria was estimated by Turbidometric immunoassay method

Principle: The reagents containing polyclonal goat antihuman Microalbumin when mixed with the urine sample containing Microalbumin cause changes in absorbance, due to the development of turbidity, which is directly proportional to the concentration of Microalbumin in the sample.

Specimen: Fresh Urine

Reference Value: 0-20mg/l

Insulin Resistance: Insulin resistance was measured by Homeostatic Model Assessment (HOMA), a method for assessing β cell function and insulin resistance from fasting glucose and insulin.

Insulin resistance was measured using HOMA 2 computerized method (137) . The blood estimation was carried out two weeks after the coronary angiogram to achieve the stability and to any prevent the changes in insulin resistance values which is caused due to the acute burden of the disease and angiographic procedure (358). In large epidemiological studies have demonstrated that, the measurement of insulin resistance by HOMA-IR has been shown to correlate well with gold standard hyper-insulinemia euglycemic glucose clamp technique (361).
Severity of Coronary artery disease: The severity of coronary artery disease was determined by SYNTAX, Gensini and Extent scoring system (362-364).

SYNTAX Score: The Syntax score was assessed by a computer program consisting of sequential and interactive self-guided questions which is available online (www.syntaxscore.com) (362).

1. Dominance
2. Number of lesions
3. Segments involved per lesion
4. Total occlusion
   i. Number of segments involved
   ii. Age of the total occlusion (>3 months)
   iii. Blunt Stump
   iv. Bridging collaterals
   v. First segment beyond the occlusion visible by anterograde or retrograde filling
   vi. Side branch involvement
   vii. Trifurcation - Number of segments diseased
5. Bifurcation – Type and Angle between the distal main vessel and the side branch <70°
6. Aorto-ostial lesion
7. Severe tortuosity
8. Length >20mm
9. Heavy calcification &
10. Thrombus
11. Diffuse disease/small vessels - Number of segments with diffuse disease/small vessels.

The syntax scoring was done by an experienced cardiologist who was blind to other parameters.
**Gensini Score:** The Gensini score defined based on the percentage reduction in lumen of coronary arteries (363). It is designated as 1 for 1-25% stenosis, 2 for 26-50% stenosis, 4 for 51-75% stenosis, 8 for 76-90% stenosis, 16 for 91-99% stenosis and 32 for total occlusion (363). The score is then augmented by a factor based on the location of lesion in the coronary artery. If the lesion is located left main 5 points were given; For proximal left anterior descending (LAD) or left circumflex (LCX) artery lesion 2.5 was given, 1.5 if the lesion is located in mid segment LAD and LCX; 1 for the distal segment of LAD and LCX lesion. Similarly 1 was point was given if the lesion was seen in first diagonal branch, first obtuse marginal branch, right coronary artery, posterior descending artery, and intermediate artery. For the lesion situated in second diagonal and second obtuse marginal branches 0.5 point was awarded.

**Extent Score:** Extent Score indicates the proportion of the coronary arterial tree involved by atheroma which can be visualized by coronary angiography (364). The proportion of each vessel involved in the lesion, was multiplied by a factor depending on the lesion location on the particular artery: a score of 5 was given for left main artery, 20 for left anterior descending artery, left circumflex artery and right coronary artery. The proportion of lesion in main diagonal branch, obtuse marginal main posterior descending branch were multiplied by 10. The first septal and posterolateral vessels were multiplied by factor 5. For completely occluded vessel the proportion of vessel visualized by collateral flow was considered, and the proportion which could not be visualized was given the mean extent score of the remaining vessels. The scores for each vessel or branch were combined to give a total score out of 100, which represents the percentage of atheroma in the coronary intimal surface area (364).

**Coronary Collaterals Grading:** Coronary collaterals were graded by Rentrop’s classification of collaterals (156).

- 0 = no collateral filling
- 1 = filling of branches but not the epicardial segment
- 2 = partial filling of the epicardial segment
- 3 = complete filling of the epicardial segment distal to the occlusion
Statistical Analysis:

Data were represented as Mean ± Standard deviation or as median and inter quartile range if the distribution is skewed. The categorical variables are represented as proportions/percentages. The normality assumption for continuous variables was evaluated by the Kolmogorov-Smirnov test.

For continuous variables, Independent sample t-test was done to compare the mean difference between two groups. For categorical variables Chi Square test was performed.

1) The correlation between these parameters was assessed by calculating Pearson’s correlation coefficient.

2) The Linear regression model was used to assess association between conventional risk factors of CAD with severity of CAD. First, the univariate liner regression analysis was used to identify potential risk factors associated with severity of CAD. Then the variables with p value < 0.20 in the univariate analysis, were included in the multivariate linear regression model to find out whether there is a significant association between HOMA-IR and severity of CAD.

3) The One Way Analysis of Variance (ANOVA) was performed in order to identify the differences in the mean syntax score, vessel score, coronary collaterals and urine microalbumin between the groups. The post hoc analysis was done assuming the equality of variances. The least square difference (LSD) post hoc test was performed to find out the significant differences in the mean syntax score, vessel score, coronary collateral grading and microalbumin levels between the non-diabetic, diabetes with less than five years’ duration, five to 10 years of diabetes and more than 10 years of diabetic duration.

4) Receiver operating curve (ROC) was plotted to find out the optimal cut-off value for HOMA-IR and fasting insulin to predict complex and severe CAD (SYNTAX > 22) and MACE.

5) Multivariate logistic regression analysis was done to identify the variables that were independently associated with a syntax score of above 22 and for predicting No Apparent Coronary Artery disease. The adjusted odds ratios for Insulin resistance, fasting insulin and
other biochemical markers were estimated, and the results were given as adjusted odds ratio (OR) and 95% CI.

6) Manipal Diabetes Coronary Artery Severity Score (MDCASS 2): A computer generated randomization of the study population was done. Among them 2/3 of subjects were included for severity score building and 1/3 of subjects for score validation respectively. 194 subjects were studied for severity score model building and data of 96 subjects were used to validate the developed score. Multivariate logistic regression analysis was done to identify the variables that were independently associated with a syntax score of above 22. Based on logistic co-efficient (B) derived from the logistic regression, a score was assigned for each significant risk factor. The accuracy of the scoring system and its validation was determined by calculating the area under receiver operating characteristic (ROC) curve and its 95% confidence intervals. The accuracy of cut-off point was determined by specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV). Using the ROC curve the cut-off point for predicting the syntax score above 22 was selected such that the NPV was at least 90%. Likelihood ratio and 95% confidence intervals for specificity, sensitivity were also calculated.

7) Cox proportional hazard model was used to assess risk factors for adverse events. First, the univariate cox proportional was used to identify potential predictors of adverse cardiac events at one year. Then the variables with p value < 0.20 in the univariate analysis, were included in the multivariate cox proportional hazard model to identify the potential predictors of adverse events at one year. Time to events were summarized and displayed using cumulative incidence curve by Kaplan-Meier survival analysis method

All statistical analysis was performed using statistical package for social sciences [SPSS] version 15. A p value < 0.05 was considered to be statistically significant. The graphical representation of hazard ratio for MACE, odds ratios for severe and complex CAD by versus insulin deciles was plotted using Sigma plot version 11.