MATERIAL AND METHODS

The present case-control study was done in Amritsar. All the patients were recruited from three heart and diabetes clinics at Amritsar city: A.P. Hospital (Ajit Nagar), Mata Kaulan Hospital (East Mohan Nagar), Dr. Puneet Arora’s diabetes clinic and research centre (Green Avenue) as shown in Figure 5. The study was done on a total of 300 subjects including 100 (50 males and 50 females) type 2 diabetes mellitus (T2DM) patients with coronary artery disease (CAD), 100 (50 males and 50 females) T2DM patients without CAD, 50 (25 males and 25 females) CAD patients without any history of diabetes and 50 (25 males and 25 females) age and sex matched healthy subjects. Diagnosis of T2DM was based on the criteria established by WHO (1999) i.e. fasting blood glucose ≥110 mg/dl. The diagnosis of CAD was based on the following criteria: a) definite history of an episode of myocardial infarction b) definite history of angina pectoris with documented electrocardiogram (ECG) findings and under specific therapy c) electrocardiographic findings, namely the Minnesta codes 1-1, 4-1, 5-2 or 9-2. All the diagnoses were made by the physician. Only patients with newly diagnosed CAD were included in this study. Healthy subjects were selected by random selective sampling from different areas in and around Amritsar. The inclusion criteria for healthy subjects were normal glucose tolerance, absence of angina, myocardial infarction or history of any vascular disease and a normal resting 12-lead ECG. The non-diabetic subjects were taken as control group for comparison with diabetic subjects.

Patients on anti-inflammatory drug therapy or with a history of cancer were not included in the study sample. Individuals suffering from renal disease, thyroid disorder, rheumatoid arthritis and acute infections were also excluded. The present study was approved by the ethical committee of Guru Nanak Dev University, Amritsar. Written consent was obtained from each subject after explaining the
objectives as well as methodology of the study. Copy of the consent form has been given in Annexure I.

A comprehensive survey of literature was done from various research journals to design the proforma. A detailed proforma, including physical activity and socioeconomic status questionnaire, for taking up patient history was designed. Copy of the proforma has been given in Annexure II. A proforma-based interview was used to collect information about habitat, sociodemographic variables i.e. current age (age at the time of last examination), disease history: age at diagnosis of diabetes (age when the diagnosis of diabetes was first recorded by a physician), duration of diabetes (period between age at diagnosis and current age), family history of CAD (myocardial infarction or sudden death before the age of 55 years in father or any other male first-degree relative, or before the age of 65 years in mother or any other female first-degree relative). Smoking and alcohol consumption habits were also recorded.

Global Physical Activity Questionnaire according to WHO (2004) recommendation was used to determine the physical activity level (Annexure II). Data on socioeconomic status was collected according to the criteria of Aggarwal et al. (2005). The questionnaire for physical activity and socioeconomic status was slightly modified according to the requirement of the present study.

In the construction of the proforma, utmost care was taken to make it broad based, so that all the aspects desired to be studied could be incorporated in its body. During data collection, personal interview was held with each subject. Interview method has been considered to be more appropriate as it provides an opportunity to the interviewer to be able to extract the appropriate information by coming in face to face contact with the subject. Before conducting the actual interview, a sample recheck from 50 subjects was carried out to ascertain the validity of the collected information.
For determining obesity and body composition, following anthropometric measurements were taken on each subject using the standard technique given by Weiner and Lourie (1981).

Body Height
Body Weight
Waist Circumference
Hip Circumference
Biceps Skinfold
Triceps Skinfold
Subscapular Skinfold
Suprailiac Skinfold

Detailed technique for each measurement has been given in Annexure III.
**Body composition** was also determined by a body fat analyser (BodyStat1500) using bioelectric impedance analysis (BIA). The body fat analyzer measures the flow of electric signals as they pass through fat and lean areas and water in the body. Fat is a bad conductor of electricity where as the lean mass and water is a good conductor. When the amount of fat and lean matter or water changes, so do the signals, giving a reliable and accurate measure of the amount of each of these components that make up the total weight of the person. Detailed technique for the measurement of body composition has been given in Annexure III.

For **blood pressure** measurements, the recommendations of Rose and Blackburn (1968) were followed using standard auscultatory method. Three readings were taken five minutes apart and their means were used in subsequent analysis. The technique for measurement of blood pressure has been given in Annexure III. All the instruments were calibrated and verified before they were used. The measurements were taken single handedly by the investigator herself.

Participants were required to fast at least 12 hours before the blood samples were taken. 5ml of blood sample was collected from each subject and the sample was divided into two parts and put in separate centrifuge tubes; one containing anticoagulant 0.5M EDTA (for plasma) and the other without anticoagulant (for serum). Serum was separated by centrifugation of blood sample without anticoagulant within 3 hours of collection. Serum was removed from the cellular material and used for determination of lipid profile. Plasma was separated from EDTA-blood samples by centrifugation at 2500 rpm for 10 minutes and stored at –20°C for analysis of monocyte chemoattractant protein-1 (MCP-1) and dimeric pyruvate kinase M2 (dM2-PK).

**Analysis of blood**

Estimation of serum lipid profile

Estimation of MCP-1

Estimation of dM2-PK
Serum lipid profile

Serum obtained from blood sample of each subject was used for the estimation of lipid profile [cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol, triglyceride] using automated blood analyzer (Vitros DT60 Chemistry System). Detailed technique for the determination of lipid profile has been given in Annexure IV.

Cholesterol

The assay used for the measurement of cholesterol was based on enzymatic method given by Allain et al. (1974). A drop of supernatant was deposited on the VITROS CHOL DT slide. The Triton X-100 surfactant in the spreading layer helped in dissociating the cholesterol and cholesterol esters from lipoprotein complexes present in the sample. Hydrolysis of the cholesterol esters to cholesterol was catalysed by cholesterol ester hydrolase. Free cholesterol was then oxidized in the presence of cholesterol oxidase to form cholesterol and hydrogen peroxide. Hydrogen peroxide oxidized a leuco dye in the presence of peroxidase to generate a coloured dye. The density of dye formed was proportional to the concentration of cholesterol in the sample and was measured by reflectance spectrophotometry. Detailed technique for the estimation of serum cholesterol has been given in Annexure IV.

Triglyceride

The analysis was based on enzymatic method, as described by Spayd et al. (1978). A drop of serum sample was deposited on the VITROS TRIG DT slide. The Triton X-100 surfactant in the spreading layer helped in dissociating the triglyceride from the lipoprotein complexes present in the sample. The triglyceride molecules were then hydrolysed by lipase to yield glycerol and fatty acids. Glycerol diffused to the reagent layer, where it was phosphorylated by glycerol kinase in the presence of adenosine triphosphate (ATP). In the presence of L-α-glycerol-phosphate oxidase, L-α-glycerophosphate was then oxidized to dihydroxy acetone phosphate and hydrogen peroxide. Hydrogen peroxide oxidized a leuco dye in the presence of peroxidase to generate a coloured dye. The density of dye formed was proportional to triglyceride concentration in the sample and was measured by reflectance spectrophotometry.
Detailed technique for the estimation of serum cholesterol has been given in Annexure IV.

**High density lipoprotein cholesterol**

HDL-cholesterol was separated by precipitation of LDL and VLDL using dextran sulphate and magnesium chloride, as described by Warnick *et al.* (1982). The HDL lipoproteins remained in the supernatant after centrifugation. The supernatant was used for further analysis. A drop of supernatant was deposited on the VITROS HDLC DT slide. The Triton X-100 surfactant in the spreading layer helped in dissociating the cholesterol and cholesterol esters from lipoprotein complexes present in the sample. The HDL-cholesterol was then hydrolysed and oxidized in the presence of cholesterol oxidase to form cholesterol and hydrogen peroxide. Hydrogen peroxide oxidized a leuco dye in the presence of peroxidase to generate a coloured dye. The density of dye formed was proportional to the concentration of HDL-cholesterol in the sample and was measured by reflectance spectrophotometry.

**Very low density lipoprotein cholesterol**

VLDL-cholesterol concentration was calculated by using the formula given by Friedewald *et al.* (1972):

\[
\text{VLDL} = \frac{\text{Triglyceride}}{5}
\]

**Low density lipoprotein cholesterol**

LDL-cholesterol concentration was calculated by using the formula:

\[
\text{LDL-cholesterol} = \text{Cholesterol} - (\text{HDL-cholesterol}) - (\text{VLDL-cholesterol})
\]

**Estimation of Monocyte Chemoattractant Protein-1**

For the estimation of plasma MCP-1 concentration, sandwich enzyme linked immunosorbent assay (ELISA) was performed on the stored plasma sample of each subject using ELISA kit (R&D Systems Inc. USA; Cat No. DCP00). A monoclonal antibody specific for MCP-1 was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any MCP-1 present was bound by the immobilized
antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for MCP-1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of MCP-1 bound in the initial step. The colour development was stopped and the intensity of the colour was measured at 450 nm on automated microplate reader (BioTek instruments Inc., USA). The protocol for MCP-1 ELISA has been given in Annexure V.

Estimation of dimeric Pyruvate Kinase M2

For the determination of plasma dM2-PK concentration, sandwich ELISA was performed on the stored plasma sample of each subject using ELISA kit (Schebo Biotech, Germany). ELISA plate was precoated with monoclonal antibody specific to dM2-PK. dM2-PK from the sample was bound by the antibody and immobilized on the plate. A second monoclonal antibody, which was biotinylated, was added for binding to dM2-PK during the next incubation. Then the conjugate of peroxidase and streptavidin was bound to the biotin moiety. The peroxidase oxidized tetramethylbenzidine and the concentration of oxidized tetramethylbenzidine (which was proportional to the amount of dM2-PK) was determined photometrically at 450 nm using automated microplate reader (BioTek instruments Inc., USA). The protocol for dM2-PK ELISA has been given in Annexure V.

Analysis of data

Assessment of smoking habits and alcohol consumption

On the basis of smoking habits, the subjects were classified in three categories using the criteria given by Singh et al. (2006): never smokers (individuals who had never smoked), former smokers (individuals who smoked earlier but had left smoking for at least past one year) and current smokers (individuals who were currently smoking). On the basis of alcohol consumption, the subjects were divided into three categories according to the criteria of Singh et al. (2006): never alcoholics (individuals who had never consumed alcohol), former alcoholics (those who consumed alcohol earlier but had left alcohol consumption for at least past one year) and current alcoholics (individuals who were currently ingesting alcohol).
Assessment of Physical Activity Status

Assessment of physical activity level was done using standard equations based on Metabolic Equivalent (MET). MET is the ratio of the work metabolic rate to the resting metabolic rate. One MET is defined as 1 kcal/kg/hour and is equivalent to the energy cost of sitting quietly.

Total Physical Activity MET minutes per week

\[= [(P2 \times P3 \times 8) + (P5 \times P6 \times 4) + (P8 \times P9 \times 4) + (P11 \times P12 \times 8) + (P14 \times P15 \times 4)]\]

(*refers to multiplication; P1-P15 refer to the question numbers listed in the physical activity questionnaire in Annexure II)

Physical activity was divided into three categories: high, middle and low as shown in Table 7

**Table 7: Criteria for assessment of physical activity level**

<table>
<thead>
<tr>
<th>Physical activity level</th>
<th>Criteria</th>
</tr>
</thead>
</table>
| High                   | IF: (P2 + P11) >= 3 days AND Total physical activity MET minutes per week was ≥ 1500  
                          OR  
                          IF: (P2 + P5 + P8 + P11 + P14) >= 7 days AND total physical activity MET minutes per week was ≥ 3000 |
| Moderate               | IF: (P2 + P11) >= 3 days AND ((P2 * P3) + (P11 * P12)) ≥ 60 minutes  
                          OR  
                          IF: (P5 + P8 + P14) >= 5 days AND ((P5 * P6) + (P8 * P9) + (P14 * P15) ≥ 150 minutes  
                          OR  
                          IF: (P2 + P5 + P8 + P11 + P14)>= 5 days AND Total physical activity MET minutes per week ≥600 |
| Low                    | IF: the value did not reach the criteria for either high or moderate levels of physical activity |

*multiplication, P1-P15: question numbers listed in the physical activity questionnaire in Annexure II, MET: Metabolic Equivalent
Assessment of Socioeconomic Status

The proforma for the assessment of socioeconomic status consisted of 22 questions (Annexure II). Suitable weightage was given to each question and scoring for each question was based on a scale ranging from 3 to 9, as recommended by Aggarwal et al. (2005). The maximum aggregate score was 100. Based on the final score, the socioeconomic status of the family was divided into three socioeconomic categories: high (score >60), middle (score 30-60) and low (score<30)

Assessment of Obesity

Obesity was assessed according to body mass index (BMI), waist circumference, Waist-Hip Ratio (WHR) and percent body fat as shown in Table 8.

BMI and WHR were calculated from the following equations:

\[
\text{BMI} = \frac{\text{Weight (kg)}}{\text{Height}^2 (m)}
\]

\[
\text{WHR} = \frac{\text{Waist Circumference (cm)}}{\text{Hip Circumference (cm)}}
\]

Estimation of body fat was done by using the table derived from the equation of Durnin and Womersley (1974) as given in Annexure III. From this table, percent body fat could be read off corresponding to differing values for the sum of four skinfolds (biceps, triceps, subscapular, suprailiac).

Table 8: Standards used for the assessment of obesity

<table>
<thead>
<tr>
<th>Obesity variable</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>≥23 kg/m²</td>
<td>WHO (2000)</td>
</tr>
<tr>
<td>WC</td>
<td>≥85 cm for males, ≥80 cm for females</td>
<td>Snehalatha et al. (2003)</td>
</tr>
<tr>
<td>WHR</td>
<td>≥0.89 for males and ≥0.81 for females</td>
<td>Snehalatha et al. (2003)</td>
</tr>
<tr>
<td>Percent body fat</td>
<td>&gt;25 for males and &gt;30 for females</td>
<td>WHO (2000)</td>
</tr>
</tbody>
</table>
Assessment of hypertension

The hypertension status of study subjects was assessed according to the guidelines of Joint National Committee on the prevention, detection, evaluation and treatment of high blood pressure (JNC VII), as reported by Chobanian et al., (2003). Subjects having SBP ≥140 mm Hg and DBP ≥90 mm Hg were classified as hypertensive.

Assessment of dyslipidemia

Hypercholesterolaemia was diagnosed in the presence of serum cholesterol >200mg/dl. Table 9 shows the reference value of cholesterol, as recommended by NCEP III (2001).

Table 9: Reference values for serum cholesterol

<table>
<thead>
<tr>
<th>Category</th>
<th>Cholesterol concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desirable</td>
<td>&lt;200*</td>
</tr>
<tr>
<td>Borderline High</td>
<td>200-239</td>
</tr>
<tr>
<td>High</td>
<td>&gt;240</td>
</tr>
</tbody>
</table>

*According to NCEP III (2001)

Hypertriglyceridaemia was diagnosed in the presence of serum triglyceride >150mg/dl. Table 10 shows the reference value of triglyceride, as recommended by NCEP III (2001).

Table 10: Reference values for serum triglyceride

<table>
<thead>
<tr>
<th>Category</th>
<th>Triglyceride concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;150*</td>
</tr>
<tr>
<td>Borderline High</td>
<td>150-199</td>
</tr>
<tr>
<td>High</td>
<td>200-499</td>
</tr>
<tr>
<td>Very High</td>
<td>≥500</td>
</tr>
</tbody>
</table>

*According to NCEP III (2001)
Low HDL-cholesterol was defined as serum HDL-cholesterol < 40 mg/dl. Table 11 shows the reference values for HDL-cholesterol, according to the recommendation of NCEP III (2001).

Table 11: Reference values for serum HDL-cholesterol

<table>
<thead>
<tr>
<th>Category</th>
<th>HDL- cholesterol concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>&lt;40*</td>
</tr>
<tr>
<td>Normal</td>
<td>40-59</td>
</tr>
<tr>
<td>High</td>
<td>≥60</td>
</tr>
</tbody>
</table>

*According to NCEP III (2001)

Statistical analysis

Statistical Package for Social Sciences 16.0 (SPSS 16.0, SPSS Inc, Chicago III) was used for statistical calculations. The results for continuous variables were presented as means ± standard deviation (SD) or percentage. The statistical significance of intergroup differences was analyzed by Student’s t-test (two tailed for continuous data) and the chi-square test (for discrete data). One-way analysis of variance (ANOVA) was used for comparison of means of more than two groups. p-value <0.05 was taken as the level of significance. Pearson’s correlation analysis was used to study the association of MCP-1 and dM2-PK with various CAD risk factors. Multivariate linear regression analysis was performed to find out the factors which were independently associated with plasma MCP-1 and plasma dM2-PK concentration. Sensitivity and specificity of plasma MCP-1 and dM2-PK as markers of CAD was also calculated.

For the determination of CAD risk factors, univariate regression analysis was performed to establish the factors significantly associated with CAD. Multiple logistic regression analysis was also performed (using CAD as the dependent variable and the risk factors, which had a significant association with CAD in the univariate analysis, as independent variables) in order to determine the independent risk factors for CAD. Odds ratio (OR) and 95% confidence interval (95% CI) was calculated for each risk factor. The detailed formulae used in statistical calculations have been given in Annexure VI.