3.1 **Study Population**

3.1.1 **Events Definitions and Exclusion Criteria**

A total of 167 patients with type 2 diabetes were selected from patients who regularly attend the Cardiology and Endocrinology Clinics at the University of Malaya Medical Centre. Of these, eighty one patients suffered from coronary artery disease (CAD), whilst eighty six are without CAD. In addition, seventy seven CAD patients without T2DM were also included in this study. In all diabetic patients, the diagnosis of type 2 diabetes was made by a consultant in internal medicine based on clinical and laboratory analyses. T2DM was considered to be present if the fasting blood glucose was ≥7.0 mmol/l, the 2-hour blood glucose in the oral glucose tolerance test (OGTT) was ≥11.1, and/or use of glucose-lowering medication (World Health Organization, 1999). T2DM patients without CAD were required to have normal electrocardiogram (ECG) and no history or symptoms of CAD (i.e., history of myocardial infarction or angina pectoris) exhibited by the patient. T2DM patients with abnormal ECG results were not included in the T2DM patients without CAD group.

The procedures used for evaluation of coronary artery disease were adapted from those previously described by Lamarche and co-workers (1995). The diagnosis of CAD was made clinically on the basis of a history of myocardial infarction (MI) and/or from a review of resting ECGs with Q wave changes suggestive of MI, coronary artery bypass
grafting, positive exercise-tolerance test, or coronary angiography (cardiac catheterization). However, the selection of the appropriate test depends on the symptoms exhibited by the patient. The decision to perform cardiac catheterization was made after having assessed the effects of pharmacological treatment by means of a further exercise test, or more rapidly when a severe disease is suspected on the basis of the first test result.

All diabetic patients were under dietary therapy and/or prescribed oral hypoglycemic agents such as sulphonylureas and/or metformin. Subjects of age $\geq 70$ years, those with known hepatic diseases, with peripheral vascular disease as determined by ankle-brachial index, history of peripheral vascular surgery, symptoms of claudication (i.e. leg pain), or under antioxidants medications were excluded from this study. Smokers and former smokers were also excluded and none of the patients had retinopathy, nephropathy, neuropathy, or erectile dysfunction. Furthermore, premenopausal women were not included since estrogens affect plasma lipids and susceptibility of LDL to oxidation (Maziere, et al., 1991). As a control, 104 age-, and sex-matched healthy subjects were recruited from Klang Valley, government bodies such as Police Force, and private centers in Kuala Lumpur. Information was acquired through the distribution of questionnaires. Healthy control subjects were free from any vascular diseases or any increase in glucose levels or both. The exclusion criteria for patients were also applied for control group.

### 3.1.2 Blood Sampling and Study Design

Blood samples were taken in the morning between 8:00 and 9:00 a.m after an overnight (12-14 hours) fast from each patient and from healthy control subjects. Samples were collected from each participant in two tubes, 5 ml in EDTA (1 mg/ml) tube and 5 ml
in anticoagulant-free tube. Of the 5 ml EDTA anticoagulated blood, 1 ml was for hemoglobin A1c (HbA1c) analysis. Blood samples were centrifuged at 3000 g for 15 minutes at room temperature within 90 min after collection and the supernatant was stored at -80°C for MDA assay and -20°C for other assays (Holvoet, *et al.*, 1998). Written informed consent was obtained from all patients and control subjects prior to their inclusion in the study. The study was performed in accordance to the approval given by the Faculty Perubatan University of Malaya Medical Ethics Committee (FPUM MEC). The experimental protocol for anti-sera preparation was approved by the local ethics committee for animal experimentation in the Faculty of Medicine, University of Malaya.

All samples were subjected for routine biochemical analysis including glucose, HbA1c, total cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol. In addition, Nε-(carboxymethyl)lysine (CML), as a common advanced glycation end product which appears in both glycation and lipid peroxidation, were quantitated in all patients and control subjects by using competitive ELISA employing polyclonal anti-CML antibodies raised in New Zealand rabbit. Occurance of lipid peroxidation in terms of malondialdehyde (MDA) was confirmed by the thiobarbituric acid–reactive substances (TBARS) colorimetric assay. Plasma samples were used to isolate LDL fraction to be used for further studies. The LDL fractions of lipoproteins were separated, dialyzed, filter sterilized, modified, and labeled by fluorescent dye to be used for metabolic studies in human hepatoma (HepG2) cell line. The effects of *in vivo* and *in vitro* modifications of LDL on its metabolism were compared.
3.2 **General Biochemical Analysis**

Glucose levels were measured by using the hexokinase-glucose-6-phosphate dehydrogenase UV-method (Kunst, *et al.*, 1983). HDL cholesterol, triglyceride and total cholesterol levels were measured by enzymatic methods using Dimension Clinical Chemistry System (Dade Behring Inc., Newark, USA). LDL cholesterol was calculated with the Friedewald formula (Friedewald, *et al.*, 1972). Percent HbA1c was determined by COBAS INTEGRA systems (Roche diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland) according to the manufacturer’s instructions. Briefly, total Hb and HbA1c concentrations were determined after hemolysis of the EDTA anticoagulated whole blood specimens. HbA1c was measured by immunoturbidimetric method using latex bead monoclonal antibodies while total Hb was measured colorimetrically. The ratio of both concentrations yields the final percent result as HbA1c percentage.

3.3 **Preparation of Glycation-Modified Proteins**

3.3.1 **CML-Modified Proteins**

CML-bovine serum albumin (CML-BSA) and CML-hemoglobin (CML-Hb) were prepared as described previously (Reddy, *et al.*, 1995; Ikeda, *et al.*, 1996). Briefly, BSA or Hb (50 mg/ml in each case) was mixed with 45 mM glyoxylic acid and 150 mM Na(CN)BH$_3$ in 10 ml of 0.2 M sodium phosphate buffer (pH 7.4). The mixture was incubated at 37°C for 24 h. Preparations were extensively dialyzed against 1 liter of 0.02 M phosphate-buffered saline (pH 7.4) to remove free glucose at 4°C with stirring (using a magnetic stirrer) with 3 buffer changes at 6 hours intervals. Controls (i.e. unmodified proteins) were prepared by the same way as above with the exception that glyoxylic acid was not included in the reaction mixture. At the end of the glycation process, the
preparations were filter sterilized by passing through a 0.45-µm filter and protein concentrations were measured by the Bradford method (1976).

3.3.2 **CEL-Modified Proteins**

CEL-BSA and CEL-Hb were prepared according to the method of Ahmed, *et al.* (1997). CEL-modified proteins were prepared by overnight incubation of 50 mg/ml of BSA or Hb at 37°C with 45 mM pyruvic acid and 150 mM Na(CN)BH$_3$ in 2 ml of 0.2 M sodium phosphate buffer (pH 7.4) followed by dialysis and filter sterilization as above. Control proteins were prepared in the same manner, except that pyruvic acid was omitted from the reaction mixture.

3.4 **Characterization of Modified Proteins**

3.4.1 **Agarose Gel Electrophoresis**

Agarose gel electrophoresis was performed according to the method of Bjerrum and co-workers (Bjerrum, *et al.*, 1973) with modifications. Agarose gel (1.5%) suspended in a mixture of 0.1 M Tris and 0.038 M glycine buffer (pH 8.7) was melted in the microwave oven for 90 seconds before casting the gel. Each sample (8 µl) was mixed with 4 µl of loading buffer prepared by dissolving bromophenol blue (0.025 %) and 1.5 M Tris in 20% glycerol, pH 9.2. Modified and non-modified proteins (1 mg/ml) were loaded into each well of the agarose gel (50-60°C). The gel tray was run for 40-60 min at 100 V and the progress of the gel was monitored by reference to the bromophenol blue. The gel was stained with 1% Coomassie blue in methanol-acetic acid-water (1:4:5) for 1 hour and destained overnight by destaining in a solution containing methanol-acetic acid-water (1:1:8).
3.4.2 **Trinitrobenzenesulfonic Acid Assay**

The degree of modification of lysine residues was quantitatively monitored by the trinitrobenzenesulfonic acid (TNBS) assay (Habeeb, 1966). TNBS reacts specifically with free lysines and NH$_2$-terminal amino acid residues to form trinitrophenyl derivatives and therefore the concentration of trinitrophenyl derivatives in proteins, such as BSA, Hb, and LDL, reflects the degree of their glycation. The reaction initiated by mixing of 400 µl CML-BSA, CEL-BSA, CML-Hb, or CEL-Hb (1 mg/ml in each case) with 400 µl of 4% NaHCO$_3$ (pH 8.4) followed by adding 400 µl of 0.1% TNBS. The solution was allowed to react at 37°C for 2 h. Optical density was measured at 340 nm and the degree of glycation was calculated from the reduction of absorbance compared with unmodified proteins. Absorbance at 340 nm of control (non-modified) samples was considered as 100% and percentage of modification in glycated samples calculated based on this value.

3.5 **Production of Polyclonal Anti-CML Antibodies**

3.5.1 **Animal Immunization**

For preparation of polyclonal antibody against CML, CML-BSA (0.5 mg/ml) was emulsified in an equal volume of 50% Freund’s complete adjuvant in total volume of 2 ml and injected intramuscularly into multiple sites in female New Zealand white rabbit (2.5 kg weight). Adjuvant is combined with the antigen to improve the immune response so that less vaccine is needed to produce more antibodies. The adjuvant allows a slow release of the antigen which allows for continual stimulation. This procedure was repeated at weekly intervals for 3 weeks. After a 2-week pause, the rabbit was received three booster injections at 2-week intervals with the same amount of immunogen.
emulsified in an equal volume of 50% Freund’s incomplete adjuvant (Hamelin, *et al*., 2003). Antibody response was monitored weekly by Ouchterlony double diffusion assay.

### 3.5.2 Immunodiffusion Analysis

To monitor the presence of anti-CML antibodies, Ouchterlony double diffusion (ODD) procedure (Ouchterlony, 1964) was performed in 60-mm plastic petri dish containing 4 ml of a 1% (w/v) agarose gel prepared in phosphate-buffered saline (pH 7.4). Agarose was boiled until fully dissolved. The temperature was decreased to 50-60°C and 4 ml volumes were dispensed into the plastic petri dish. The plate was cooled for 5 min uncovered and for an additional 20 min covered. To perform the immunodiffusion test, five wells, one in the center and the other four at the sides about 0.6-0.8 cm apart from the central well, were made in the gel by hand using a 3-mm diameter plastic punch after placing the petri plate over a paper template as a guide. The center well was filled with 25 µL anti-CML antibodies and the surrounding wells with 25 µL of antigens (CML-BSA, CML-Hb, and BSA) and allowed to react in a moist chamber for 48-72 hours at 4°C to obtain highest resolution. The gel was washed for 24 h by submerging gels in PBS. The gel was then dried, stained with 0.5% Coomassie Brilliant Blue (w/v) in methanol, water, and acetic acid (4:5:1), destained using the mixture containing methanol:water:acetic acid (4:5:1), and photographed. The antiserum showed a single precipitin line with CML-BSA and CML-Hb upon a double immunoprecipitation analysis whereas BSA showed no reaction with antiserum. The rabbit was then bled on the tenth day after the last booster injection and antiserum was obtained to be used for ELISA.
3.6 Antiserum Titration and Characterization

3.6.1 Antiserum Titre Determination

Antiserum titre was determined by means of non-competitive ELISA using CML-BSA as coating antigen. Antiserum titre was defined as the serum dilution giving a 50% maximum absorbance (405 nm) signal employing horseradish peroxidase (HRP)-conjugated anti-rabbit IgG as secondary antibody (Makita, et al., 1992). Each well of a 96-wells plate was coated with 100 µl of CML-BSA (5.0 µg/ml) in 50 mM carbonate/bicarbonate buffer (pH 9.6). After 1 h incubation, the plate was washed three times with 200 µl of a washing solution containing PBS, 0.05% Tween 20 (buffer A) and unoccupied sites on the wells were blocked with 100 µl of 6% (w/v) skimmed milk in PBS for 1 h with shaking. Wells were washed three times as above and then 100 µl of antiserum, serially diluted (1:10^2-1:10^5) in dilution buffer prepared by mixing of 0.02% Tween 20 with PBS (buffer B) was added to each well and incubated for 1 h with shaking. The plate was then washed and incubated for 1 h with shaking with 100 µL HRP-conjugated anti-rabbit IgG (0.1 µg/ml) followed by reaction for 30 min with 50 µl of 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate at room temperature in the dark. The reaction was terminated by adding 100 µl of 1.0 N H_2SO_4 and the absorbance at 405 nm was measured on an ELISA reader (MRX Microplate Reader, Dynatech Laboratories Ltd, UK).

3.6.2 Characterization of Polyclonal Antibodies to CML

The cross-reactivity of polyclonal anti-CML antiserum with several proteins (CEL-BSA, CEL-Hb, BSA, Hb) and competitors (CML-BSA, CML-Hb) was evaluated by competitive ELISA at room temperature. CML-BSA (used as adsorbed ligand antigen)
and the proteins and competitors diluted with buffer B into different concentrations (0.250, 0.500, 1.0, 5.0, 20, 50, 100 and 250 µg/ml) were used in the experiment. Each well of an immunoplate was incubated with 100 µl of CML–BSA (1.0 µg/ml) in 50 mM carbonate buffer (pH 9.6) at room temperature for 1 h. The wells were washed three times with buffer A and blocked with 100 µl of 6% skimmed milk in PBS for 1 h with shaking followed by triple washing as above. Each protein or competitor to be tested (60 µl) was mixed with 60 µl of antiserum (final dilution 1:2000) for 1 h and the mixture (100 µl) was added to the wells of the microtiter plate followed by shaking for 1 h. The wells were washed again, and 100 µL HRP-conjugated anti-rabbit IgG (0.1 µg/ml) was added to each well. After incubation for 1 h at room temperature, the wells were washed and color was developed after incubation with 50 µl substrate solution containing 2,2’-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid (ABTS). The wells were incubated for 20-30 min at room temperature in the dark followed by addition of 100 µl of 1.0 N H₂SO₄ to stop the reaction. The absorbance at 405 nm was read using an ELISA reader (MRX Microplate Reader, Dynatech Laboratories Ltd, UK).

3.7 **CML Measurement by Competitive ELISA**

CML in the serum of the patients and healthy control subjects were measured by competitive ELISA as described above except that after incubation of the wells with the blocking solution (6% skimmed milk), 60 µl serum (from patients/controls) or CML-BSA standard with serial dilutions (0.100, 0.250, 0.500, 0.750, 1.0, 2.5, 5.0 and 7.5 µg/ml) were mixed with an equal volume of antiserum (final dilution 1:2000) for 1 h and the mixture (100 µl) was added to the wells followed by shaking for 1 h. The remaining steps were performed in the same manner to that described above and the CML concentrations
in the samples were determined from the standard curve. The CML concentration was expressed as ng CML/ml. All ELISA determinations were performed in duplicates.

3.8 Detection of Malondialdehyde

The amount of plasma malondialdehyde (MDA), an index of lipid peroxidation, was determined by the thiobarbituric acid reactive substances (TBARS) method as previously described (Ohkawa, et al., 1979). Briefly, 100 µl plasma or MDA standard was combined with an equal volume of 8.1% sodium dodecyl sulfate. Then, 2.5 ml TBA/buffer (prepared by dissolving of 0.53% thiobarbituric acid in 20% acetic acid as adjusted to pH 3.5 with NaOH) was added to the reaction mixture. The tubes were capped and incubated at 95°C for 60 min. The reaction was stopped by placing tubes on ice followed by centrifugation at 4000 rpm for 10 min. The optical density of pink color developed in the supernatant was measured against distilled water as blank at 532 nm. The measurements were performed in duplicates and the concentration of plasma MDA was expressed in µmol/l.

MDA standard was prepared from acid hydrolysis of 1, 1, 3, 3-tetraethoxypropane (TEP) according to the method of Tsaknis with modifications (Tsaknis, et al., 1998). MDA was liberated by incubating 0.3 ml of TEP with 0.3 ml of 0.2 N HCl at 95°C for exactly 5 min. After cooling on ice, the mixture was diluted to 3 ml with deionized H2O. This stock-standard solution, prepared daily, was used for preparation of standards of malondialdehyde (1.25, 2.5, 5, 8 and 10 µmol/l).
3.9 **Low Density Lipoprotein Isolation and Preparation**

EDTA-containing plasma samples were recovered by centrifugation and pooled into 28 separate pools (nine from type 2 diabetic patients with CAD, ten from type 2 diabetic patients without CAD, and nine from healthy subjects). The pooled plasma samples were then used to isolate LDL, with each sample representing six or seven patients. LDL isolation, modification, and cell culture procedures described in the next subsections were done with LDL prepared from aliquots of these plasma pools. LDL was isolated as described by Chung, *et al.* (1986). Briefly, plasma (3 ml) from each pool was adjusted to a density of 1.21 g/ml by addition of 0.981 g of KBr. Then 7 ml isotonic saline (density 1.006 g/ml) containing 1 mM EDTA was layered on top of the plasma in a Quick-seal polyallomer tube (Sorvall instruments, Thermo Electron Corp., USA). LDL was isolated by density gradient ultracentrifugation at 4°C and 65000 rpm (397000 g) for 3 hours using a fixed-angle Sorvall T-880 rotor in Sorvall ULTRA PRO® 80 Ultracentrifuge (Sorvall instruments, Thermo Electron Corp., USA). The LDL band was carefully removed by tube slicing and dialyzed in the dark at 4°C against 150 mM NaCl solution supplemented with 0.26 mM EDTA (pH 7.4). The LDL-containing solutions were sterilized by filtration using 0.22-µm filter units. Protein levels of isolated LDL fractions were measured by the Bradford method and used in the subsequent experiments.

3.10 **LDL Modifications**

3.10.1 **Oxidation and Glycation**

Copper sulfate was used as a catalyst for oxidation based on the method described by Esterbauer, *et al.* (1989). In short, 5.0 µM CuSO₄ was added to 1 ml LDL (200 µg/ml) followed by incubation at 37°C for 24 h in the dark. At the end of the incubation period,
the lipid peroxidation was stopped by cooling samples at 4°C followed by addition of 30 µl of 1 mM EDTA. AGE-LDL was prepared by incubation of 1 ml native LDL solution (0.75 mg /ml) in PBS containing 0.01% EDTA, 100 mM Butylated hydroxytoluene (BHT) and glucose (0.2 M) in the presence of 200 mM Na(CN)BH₃ for 24 h at 37°C (Schmidt, et al., 1993).

### 3.10.2 Glycoxidation and Carboxymethylation

To prepare glycoxidized-LDL (GO-LDL), 0.75 mg /ml of native LDL (n-LDL) was incubated at 37°C with glucose (0.2 M), CuSO₄ (5.0 µM) and 200 mM Na(CN)BH₃ for 24 h. The ongoing glycoxidation process was terminated by cooling samples at 4 °C and adding 30 µl of 1 mM EDTA. Native-LDL was incubated with PBS in the absence of glucose and CuSO₄ at 37°C for 24 h. CML-LDL was prepared as previously described for the preparation of CML-BSA (Reddy, et al., 1995; Ikeda, et al., 1996) using LDL at concentration of 0.5 mg/ml. Control, unmodified LDL were prepared by the same way as above but without adding glyoxylic acid to the reaction mixture. The preparations were then filter sterilized by passing through a 0.22-µm filter and protein concentrations were measured.

### 3.11 Characterization of LDL Modifications

#### 3.11.1 Electrophoretic Mobility Rate

Relative electrophoretic mobility (REM) was determined by agarose gel electrophoresis (Noble, 1968) for all samples. A 1% agarose gel was placed in an electrophoresis cell immersed in 0.05 M barbital buffer (pH 8.6). LDL samples (8 µl) were loaded onto the gel. The gel was run at 120 V for 45 min, following which it is fixed
in 5% acetic acid in 75% ethanol for 30 min. After another 30 min of drying, gel was visualized with Oil Red O staining until well-stained distinct LDL bands were obtained. The gel was immersed in destaining solution (1 part methanol and 3 parts glycerol) for 20 min. Migration of all samples is measured as distance traveled from origin to center of the lipoprotein band. Relative electrophoretic mobility was calculated as the ratio of migration of modified LDL compared with control LDL.

### 3.11.2 Degree of Glycation and Lipoxidation

The amount of free lysine remaining after LDL modification was measured by trinitrobenzene sulfonic acid (TNBS) assay (Habeeb, 1966) as previously described in section 3.4.2. The degree of oxidative modification of LDL was estimated by measuring thiobarbituric acid reactive substances (TBARS) using MDA as a standard (Ohkawa, et al., 1979) as described in section 3.8. The results were expressed as nmol of MDA per mg of protein.

### 3.12 Susceptibility of LDL to Oxidation

Oxidation of LDL was assessed by the formation of conjugated dienes as a result of oxidation (Esterbauer, et al., 1989). Briefly, 5.0 μM CuSO₄ was added to 1 ml LDL (200 μg/ml) followed by incubation at 37°C. Absorbance was read at 234 nm, in a Cary 50 UV-spectrophotometer (Varian, Inc., USA) every 5 min for a maximum of 3 h or until the rapid phase of LDL oxidation reached a plateau. In lipid peroxidation this rapid oxidation phase is usually termed the propagation phase. The lag phase as a measure of the time during which LDL is protected from oxidation by antioxidants present in the LDL was determined. The lag phase was estimated as the incubation time corresponding
to the intersection of two lines drawn from the changes in optical density, one through the initial, slowly rising curve that corresponds to the utilization of the endogenous antioxidants and the other, a subsequent, rapidly rising curve that corresponds to the propagation phase of the LDL oxidation, when most of the fatty acids are being oxidized. The lag phase is expressed as minutes after addition of CuSO$_4$.

3.13 **LDL Labeling**

Native-LDL, ox-LDL, AGE-LDL, GO-LDL, and CML-LDL were labeled with the fluorescent lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes Inc., Eugene, OR) by a modification of the method described by Stephan and Yurachek (1993). A stock solution of DiI was prepared by dissolving 3 mg DiI in 1 ml of dimethyl sulfoxide (DMSO). 50 µl of DiI stock solution was added to 1 ml of LDL solution (300 µg/ml) followed by incubation in the dark at 37°C for 16 h. Excess DiI was removed by dialysis in the dark at 4°C against 150 mM NaCl solution containing 0.26 mM EDTA (pH 7.4). DiI-LDL was then passed through a 0.22 µm filter and stored at 4°C in the dark until use, normally within 2 weeks of preparation.

3.14 **Cell Culture Experiments**

3.14.1 **HepG2 Cells Subcultures**

Human Hepatoma (HepG2) cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in three sterile tissue 25-cm$^2$ flasks in complete medium containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin
and 100 µg/ml streptomycin) in 95% air/5% CO₂ at 37°C. The cell medium was replaced on the third day with 5 ml of fresh complete medium. By day 6, the growth media was removed and adherent cells were washed twice with 1x PBS and detached by incubation of cells with 2 ml of a trypsin (2.5 g/l)/EDTA (1 g/l) for 4-5 min at 37°C. Residual trypsin activity was inhibited by addition of 3 ml of complete media after complete detachment of the cells was verified under the microscope. The cell suspension was mixed vigorously and cells were removed to a 50-ml cell culture tube. Cells were centrifuged at 1000 rpm for 5-10 min, at 17°C in a precooled centrifuge. After removing of the supernatant, cells were resuspended by adding 4 ml complete media and the cell suspension was passed several times through a fine Pasteur pipette to disaggregate cell clumps.

3.14.2 Cells Counting and Viability Testing

A mixture of 10 µl of cells suspension and 10 µl of 0.04% trypan blue was pipetted onto a hemacytometer for determination of the number of cells per volume and percentage of viable cells. This chamber contains 4 large grids, consisting of 16 smaller grids. The area of one of the large grids is 1 mm² and the height of the chamber is 0.1 mm². Hence, the volume of one large grid is 0.1 mm³ or 10⁴ cm³, which corresponds to 0.4 mm³ for the 4 large grids together. Since 1 cm³ is approximately equivalent to 1 ml, the cell concentration per ml will be the average count per square x 10⁴. The total number of cells is given by:

(Number of cells counted in the 4 large grids/4) x dilution factor x 10⁴ x volume (ml) of cell suspension.
Trypan blue is the stain most commonly used to distinguish viable from nonviable cells. Viable cells exclude trypan blue, while dead cells absorb the dye and appear blue when viewed with a microscope. Cells viability, as measured by the exclusion of 0.04% trypan blue, was greater than 92% and cells count was $2.7 \times 10^6$ cells/ml. This subculture technique was repeated until sufficient number of cells was obtained to be used in the experiments described below with a maximum of 6 passages.

### 3.14.3 Cells Freezing and Thawing

Cells may be stored for several years in liquid N$_2$ at $-170^\circ$C. Freezing cells down in their growth media can cause cellular damage due to the formation of ice crystals and changes in osmotic effects. In order to prevent this effect, a cryopreservative such as DMSO must be used. After trypsinization and centrifugation (as above), the supernatant is removed and cell pellet was resuspended by dropwise adding of 10 ml of freezing media (70% DMEM, 20% FBS, 10% DMSO) to give a final freezing density of $1.6 \times 10^7$ cells/ml followed by thorough mixing using a Pasteur pipette to avoid cells aggregation. For freezing, cells (1 ml) were transferred into 1.5 ml sterile cryotubes. Cells were frozen for 1 h at $-20^\circ$C and for 12 h at $-80^\circ$C and then stored in liquid nitrogen. Cells were thawed by placing the cryotubes in a 37°C waterbath at least one hour before use. One vial of cells was diluted into 10 ml of complete growth medium followed by gentle mixing. Cells were centrifuged down and the media above the pellet was removed. Cells were resuspended in complete growth medium and diluted to the appropriate cell density before seeding onto cell culture plates.
3.15 Native and Modified LDL Binding and Uptake

3.15.1 Spectrofluorometer Measurements

3.15.1.1 Dose-Response Assays

For quantitative measurements of DiI-LDL bound to the LDL receptor, HepG2 cells were seeded in 6-well culture plates at a density of $6 \times 10^5$ cells/well in medium containing 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). After a period of 48 h, cells were incubated with DiI-LDL with increasing concentration in serum-free DMEM at 4°C (Teupser, et al., 1996). At 4°C, only LDL bound to the LDL receptor on the cell membrane was measured because the cell system was static. In addition, other plates were incubated with prewarmed serum-free media at 37 °C in increasing DiI-LDL concentrations from 10-100 µg/ml to measure cell-associated LDL. At 37 °C, DiI-LDL can be internalized and LDL metabolism occurs because the cell system was dynamic. Cell-associated LDL (i.e. LDL uptake) constitutes LDL that is both bound and internalized within the cell system. The plates were covered and the cold plates were placed at 4°C while the others were placed in the incubator at 37°C for 2 h. After washing cells three times with PBS, 1 ml isopropanol is added to each well, and plates were gently shaken for 15 minutes. The isopropanol extract of DiI was then transferred to a glass tube, centrifuged at 3000 rpm for 15 min and the relative binding and uptake of DiI-LDL into cells was measured in the supernatant using a Cary Eclipse microplate fluorescence reader (Varian, Inc., USA) with emission and excitation wavelengths set at 548 and 574 nm, respectively. All incubations were performed in triplicates and DiI content in the extracts was calculated from the DiI standard curve. Cells were lysed by lysis buffer (1 g/l SDS in 0.1 M NaOH) for measurement of both total amount of proteins in cultured cells and internalized DiI-LDL particles. The Uptake of
modified DiI-LDL (ox-LDL, AGE-LDL, GO-LDL, and CML-LDL) and LDL particles isolated from T2DM patients with and without CAD were studied by the same way as described above except that DiI-LDL was used at a constant concentration of 20 µg/ml.

3.15.1.2 LDL Specific Uptake

To study nonspecific uptake of Dil-labeled LDL, cells were seeded in 6-well culture plates with increasing concentrations of Dil-LDL (5-100 µg/ml) in the presence of a constant concentration of 30-fold excess of unlabelled LDL. Specific uptake of Dil-LDL in all preparations was determined at a constant concentration of 20 µg labelled lipoprotein/ml with 30-fold excess of unlabelled lipoprotein. In addition, plates of cells were incubated with the same increasing concentrations of DiI-LDL in the absence of unlabelled LDL to measure total uptake. Plates were incubated at 37°C for 2 h followed by extensive washing of cells with PBS. Isopropanol was added to the cells to measure the fluorescence as described above and DiI content in the extracts was calculated from the DiI standard curve. To each well 1 ml of lysis buffer was added (1 g/l SDS in 0.1 M NaOH) and incubated at room temperature with gentle shaking in the dark for 15 min for both direct fluorescence and protein measurement. Concentration of unlabelled LDL was determined based on protein content. Specific uptake was calculated as the difference between total and nonspecific uptake. Competitive inhibition studies were carried out by incubation of cells with a constant concentration of 20 µg/ml of DiI-LDL in the presence of either 50-fold excess of unlabelled LDL or dextran sulphate, a polyanionic inhibitor of native-LDL binding and uptake, with a concentration of 20 µg/ml for 2 h at 37°C. The results were expressed as nanograms of cell-associated Dil-LDL per milligram of cell protein.
3.15.1.3 **Cell Lysates and Protein Content Determination**

Protein content of the cells was measured in duplicate after lysing of cells in lysis buffer. The concentration of protein in cell lysate was measured with the Bradford assay which relies on the binding of the Coomassie Brilliant Blue dye with protein. The dye binds more readily with the free NH$_2$-groups of arginyl and lysyl residues. An aliquot of the sample was mixed with 500 µl of the reagent and the absorption read after 5 min at 595 nm. Incubations were made against a reagent blank consisting of distilled water. Protein concentration was calculated by comparing the values of the samples with a BSA standard.

3.15.1.4 **Standard Solutions of DiI**

Standard solutions of DiI were prepared in isopropanol in the concentration range of 50–2000 ng/ml. Fluorescence measurements were performed with excitation and emission wavelengths set at 548 and 574 nm, respectively. Therefore, isopropanol was used to extract the fluorescent DiI dye from cells which allowed measurement of DiI fluorescence from the standard curve.

3.15.2 **Fluorescence Microscope Measurements**

HepG2 cells were seeded on coverslips in 6-well plates at a density of 6×10$^5$ cells/well in complete DMEM media (described above) in a CO$_2$-incubator (5% CO$_2$ in air) at 37°C. After 2 days of cell culture, cells confluency was reached. On the day of the experiment, the medium was removed and cells were washed twice with serum-free DMEM. Thereafter, 500 µl serum-free DMEM containing 20 µg DiI-LDL was added to
the cells and incubation was carried out for 2 h at 37 °C. After incubation, the cells were rinsed three times with PBS, fixed in 5% formalin in PBS, and mounted in PBS/glycerol. Cells were examined under an Olympus BX50/BX-FLA microscope (BX50; Olympus, Tokyo, Japan) equipped with a U-MNG filter set (excitation 530-550 nm, emission 590 nm) for visualization of the red fluorescence of DiI (Pitas, et al., 1983). All the images have the same exposure time and brightness/contrast settings.

### 3.16 Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Chi-square ($\chi^2$) test was used for the analysis of categorical variables. Analysis of variance (ANOVA) was used to compare the laboratory tests in the four groups of the study followed by Bonferroni-corrected repeated pair comparisons. Differences in the biochemical parameters and medications among individuals of the two diabetic groups (i.e. T2DM with and without CAD) were examined by using independent sample $t$-test. For measurements using pooled plasma, Kruskal-Wallis one-way analysis of variance (ANOVA) was used. If analysis of variance indicated that $P < 0.05$, the Mann-Whitney U-test was used to adjust the multiple comparisons. The mean difference was judged to be significant if $P < 0.05$. CML concentrations were correlated with other parameters of T2DM patients with CAD including fasting blood glucose, HbA1c, diabetes duration, lipid profile tests, blood pressure, and MDA using Pearson’s linear regression followed by multivariate regression analysis using Enter method. In healthy control, T2DM patients without CAD, and nondiabetic CAD groups, a correlation analysis between CML and other variables was also conducted.
Logistic regression analysis with *Enter* method was used to determine the magnitude of association between CML and the presence of CAD in T2DM patients. The presence of CAD was included into the model as a dependent variable and CML, fasting blood glucose, HbA$_{1c}$, LDL-cholesterol, and MDA as the independent continuous variables and associations were calculated as odds ratios (OR) with the corresponding 95% confidence intervals (CI). The WALD statistic was calculated to assess the significance of individual logistic regression coefficients for each independent variable. Statistical computations were calculated using SPSS 11.5 for Windows software (SPSS Inc, Chicago, IL, USA).