Chapter 3

3.1 Materials

All chemicals used in the study were of analytical grade. Deionized water, Carbon tetrachloride, Folin reagent was purchased Merck Chemicals, Ehrlich’s reagent (catalogue no RM-1452), NBT (catalogue no-RM-578), Skimmed milk, Chloramine-T was purchased from Himedia Laboratories Pvt., Copper sulphate from SRL Pvt Ltd. Total protein standard was purchased from Transasia Bio-Medical Ltd., Daman. Drabkin’s reagent (catalogue no Art-3602) was purchased from Loba chemicals, Microalbumin kit reagents purchased from Immulite and microalbumin calibrator were purchased from Roche Diagnostics. For routine biochemical tests kits were purchased from Eve’s Inn Diagnostics, Baroda. Heparan sulphate (catalogue no H760), DMB (catalogue no 341088) and all other chemicals were purchased from Sigma Aldrich. Human IgG, Anti human IgG HRP conjugated antibodies and TMB for ELISA were purchased from Bangalore Genei Pvt. Ltd. For detailed description of the methods we refer corresponding section.

The non-chemical and consumable items along with accessories were of high quality meant for research work. The blood samples collection vials were purchased from BD Vacutainer®, Amicon ultra filtration tube from Millipore Pvt. Ltd. Microtiter plates, storage and sample collection vials, Microtips were purchased from Tarsons Products Pvt. Ltd., Micropipette and multichannel pipettes were purchased from eppendorf and Finnpipette Thermo Scientific. The accessories and essential laboratory equipments are listed in Appendix 1.

3.2 Clinical Studies

Known type 2 diabetic patients who attended the OPD and members of diabetic club were followed up in this study. The study population consisted of 774 subjects of type 2 diabetic patients with additional 100 healthy individuals for reference.
3.2.1 Methodology
At recruitment, type 2 diabetes was defined according to standard guideline criterions. Ethical approval was sought from local ethics committees of S. K. Hospital and P. S. Medical Collage, Karamsad, Gujarat, India and informed consent was obtained from each patient as shown in Appendix 2 and 3. Standard self-administered questionnaires were used to define age, duration of diabetes, health habits (smoking, alcohol consumption and exercise), medical history and current medications as shown in Appendix 4. Height and weight were measured by using a regularly calibrated stadiometer and balance-beam scale with participants wearing light clothing and no shoes. Body mass index was calculated as weight in kilograms divided by height in meters squared. Systolic and diastolic blood pressures were measured twice by a trained nurse in seated participants after a 5-minute rest. Hypertension was defined as a systolic blood pressure (SBP) greater than or equal to 140 mm Hg or a diastolic blood pressure (DBP) greater than or equal to 90 mm Hg or under antihypertensive treatment. Follow-up questionnaires have been reported at visit of patients to OPD. With each questionnaire, we inquired about the symptom of secondary complication like vision impairment (retinopathy), numbness (neuropathy), chest pain (Macro vascular complications, heart stroke), if they answered yes we consider cases only after reconfirmed by physician and medical records of the patients. Patients reached to end stages of diabetic nephropathy were also subjected to detailed questionnaires. Cases of age more than 70 years, nondiabetic renal problems, chronic liver disease, pregnancy, heavy alcoholic, glucocorticoid medication, urinary tract infection and diabetic foot complication were excluded.

3.2.2 Blood and Urine Samples Collection
3.2.2.1 Blood Collection
Fasting blood samples were collected from subjects in a seated position after an overnight fast of 10-14 hours. The specimen was taken from the cubital fossa of the chosen arm by standard technique. The blood samples were collected in sterile BD Vacutainer® tubes (6 ml) with and without EDTA coating.
3.2.2.2 Plasma /Serum Separation
Plasma/Serum was separated by centrifugation at 1500 x g for 15 min and aliquoted and labeled properly. The samples were stored at -20 °C until analyzed for other profile.

3.2.2.3 Urine Collection
Morning urine samples from patients with no sign of urinary tract infection were collected, the spot urine samples were collected in pre-sterile vials, after first voids of urine, and were brought to our laboratory in cool condition. The samples were centrifuged at 1000 x g for 10 minute to remove all suspended particles and cell derbies aliquoted and stored in -20 °C till further use without any preservation and analysis was done within seven days. Sodium azide with a final concentration 0.04 mg/dl was added to those samples which were stored for longer duration.

3.3 Biochemical Analysis
Biochemical parameters were estimated through standard published laboratory protocols. The following biochemical investigations were carried out in routine. Plasma glucose, albumin total cholesterol, triglycerides and HDL and LDL were determined using commercially available kits. Tests were carried out as per procedures laid out in the information sheets accompanying the kits. Low density lipoprotein (LDL-cholesterol) was calculated using the Friedewald formula. Plasma glucose and creatinine analysis were performed on the same day of sample collection. All tests were performed in triplicates for consistency in results.

3.3.1 Protein Estimation by Follin Lowry Method
Solution A: 2% (w/v) Na$_2$CO$_3$ in distilled water.
Solution B: 1% (w/v) CuSO$_4$.5H$_2$O in distilled water.
Solution C: 2% (w/v) Sodium-Potassium Tartarate.
Mix solution A, B and C in the ratio of 100:1:1 which was used as working reagent.
A 100 µl plasma sample, 1 ml 1 N NaOH mixed and kept in water bath for 10 min 2 ml of working reagent added and incubated for 10 min at room temperature. Then after 100 µl Folin reagent added and incubated for 40 min at room temperature. Absorbance was taken at 750 nm. Standard total plasma protein from Transasia Bio-Medical Ltd.
(concentration 6 g/dl) was used for calibration. Plasma proteins were expressed in g/dl.

### 3.3.2 Hemoglobin

Hemoglobin was estimated by Drabkin’s method. A 10 µl of blood was added to 2.5 ml of Drabkin’s reagent and reaction mixture was incubated for 10 min at room temperature. Absorbance was taken at 540 nm against Drabkin’s reagent. Molar extinction coefficient of Drabkin’s reagent is 44 and 0.29 O.D. =12.1 g/dl hemoglobin.

### 3.3.3 Glycated Haemoglobin Estimation

Glycated haemoglobin was estimated using standard lab procedure as described by Chandalia et al 1980. Blood sample was adjusted to 10 mg/dl of haemoglobin concentration using Drabkins reagent. Then 500 µl blood was centrifuged at 1000 x g to obtain RBC pellet. The RBC pellet was washed with normal saline (0.87% NaCl) and centrifuged 2-3 times at 1000 g for 5 min each. To that 100 µl of Carbon tetra Chloride was added and the volume made 500 µl with distilled water. Vortexed vigorously and centrifuged at 1000 x g for 20 min. A 250 µl of lysate was taken from tube. To that 1750 µl normal saline and 1 ml of oxalic acid (0.3N) added. Mixed well and kept in boiling water bath for 2 hours with cotton plug. After incubation the tubes were removed and cooled to room temperature. To this 1 ml of TCA (40%) added and centrifuged at 1000 x g. 2 ml of supernatant taken in new test tube and 0.5 ml TBA (0.7%). Mixed well and kept at 37°C for 40 min. The absorbance was taken at 443/445 nm. The blank made by 2 ml distilled water and 0.5 ml TBA. Glycated hemoglobin was expressed in percentage glycation.

### 3.3.4 Creatinine Estimation

Creatinine estimation was done using principle of modified Jaffé’s reaction of alkaline Picrate, as described by Kroll et al 1986. A 200 µl of fresh sera was mixed in 800 µl of distilled water. A 100 µl of 1:10 diluted urine and 400 µl of distilled water taken. To that 0.5 ml Sodium tungstate (10 % w/v) and 0.5 ml H₂SO₄ (0.66 N) added. Centrifuged at 1500 x g for 10 min. 1 ml supernatant was aspirated and to that 0.5 ml picric acid (0.4 M), 0.5 ml NaOH (0.75 N) and 1 ml distilled water added. Mixed well and incubated at 37 °C for 20 min. Blank was prepared mixing 2 ml distilled water, 0.5 ml Picric acid
solution and 0.5 ml NaOH. Absorbance was taken at 520 nm. Creatinine concentration was expressed in mg/dl.

3.3.5 Quantitative Estimation of Urine Microalbumin
Urinary microalbumin excretion was estimated by biochemical auto analyzer “IMMULITE® 1000 Immunoassay System”. Albumin was assayed based on the principle of a Chemiluminescent enzyme assay. The fresh urine samples were analyzed as per the instruction of kit. The biochemical auto analyzer was regularly calibrated with commercially available microalbumin calibrators.

3.3.5.1 Microalbumin-to-Creatinine Ratio (UACR)
UACR, used as a measure of albumin excretion in the microalbuminuric range, was calculated by dividing microalbumin concentration ‘mg/ml’ by urine creatinine concentration ‘g/ml’ and the results were expressed in mg/g. The UACR is more convenient to perform than a 24-hr urine collection for microalbumin estimation. The urine albumin-to-creatinine ratio (UACR) cut-off point of less than 30 mg/g as recommended by the American Diabetes Association was used to define microalbuminuria.

3.3.6 Quantitative Estimation of IgG
The plasma and urinary concentration of IgGs were measured in all the samples by sandwich ELISA.

Reagents:

(i) Coating buffer: 50 mM pH 9.6
NaHCO$_3$ – 1.17g
Na$_2$CO$_3$ – 0.3 g
Volume 200 ml with water.

(ii) Phosphate Buffer Saline Tween: (PBST) 0.1M, pH 7.2
NaH$_2$PO$_4$·2H$_2$O – 3.055 g
Na$_2$HPO$_4$·2H$_2$O – 14.54 g
NaCl – 8.8 g
(ii) Blocking Buffer: 3 % Skimmed milk in PBST
(iii) Goat anti human IgG whole serum: 1: 1000 dilution
(iv) Goat anti human IgG HRP: 1: 1000 dilution
(v) TMB/H₂O₂ solution: 1: 20 dilution
(vi) Sample/Conjugate diluent: 0.1 % Skimmed milk in PBST

The microtiter plate was coated with goat anti human IgG in carbonate buffer (150 µl/well). The plates were incubated at 4°C under humid conditions over night. The wells were aspirated out and washed thrice with PBST. The coated wells were blocked with 3% Skimmed milk (150 µl/well). It was incubated at room temperature for 1 hour. The wells were washed thrice with PBST. The samples and standards were added to wells (150 µl/well) in conjugate diluent. The plates were incubated at 37°C for 1 hour. The plates were washed three times with PBST, and 150 µl/well substrate TMB/ H₂O₂ was added. After 10 minutes the reaction was stopped with 50 µl/well 1N Sulphuric acids. The absorbance was taken at 450 nm in ELISA reader (Molecular device model spectra max 190).

3.3.6.1 Immunoglobulin G-to-Creatinine Ratio (UIgGCR)
UIgGCR, used as a measure of IgG excretion in the all patients, was calculated by dividing IgG concentration ‘mg/ml’ by urine creatinine concentration ‘g/ml’ and the results were expressed in mg/g.

3.3.7 Antioxidative Enzymatic Markers
3.3.7.1 Glutathione Peroxidase (GPx)
Glutathione peroxidase activity was measured according to method of Flohe and Gunzler 1984. A 300 µl plasma was added in test tube containing 300 µl of 0.1 M phosphate buffer (pH 7.4), 200 µl of 2 mM GSH, 100 µl of 10 mM Sodium azide. 100 µl of 1.0 mM H₂O₂ was added to start the reaction. Test tubes were incubated at 37 °C for 15 min then 500 µl of 5% TCA was added to stop the reaction. Test tubes were centrifuged at 1500 x
g for 10 min. Furthermore 100 µl of supernatant was taken and added in to 700 µl of 0.1 M phosphate buffer (pH 7.4) and 700 µl DTNB containing tubes. Absorbance was measured at 420 nm. The activity was expressed in U/mg protein.

3.3.7.2 Catalase (CAT)

Catalase activity was measured according to the method of Sinha [Sinha 1972]. A 100 µl of plasma was added to the 100 µl of 0.1 M phosphate buffer (pH 7.1) and 500 µl of distilled water containing tube and incubated at 37 °C for 10 min. A 400 µl of 0.1M H₂O₂ was added to start the reaction and incubated at 37 °C for 3 min. A 200 µl of Potassium dichromate solution (5% w/v) was added to stop the reaction. Test tubes were incubated in boiling water bath for 15 min. Precipitates were separated by centrifugation at 1500 x g for 10 min and absorption of supernatant was measured at 570 nm. The activity was expressed in U/mg protein.

3.3.7.3 Superoxide Dismutase (SOD)

The superoxide dismutase activity in plasma was measured according to the method of Kakkar [Kakkar et al 1984]. Assay mixture was contained 1.2 ml of Sodium pyrophosphate (52 mM) 100 µl of Phenazine methosulphate (188 µM), and 300 µl of Nitro blue tetrazolium (300 µM) in both test and control tubes and 200 µl of plasma (controls tubes were devoid of plasma). The reaction was started by adding 200 µl of NADH (780 µM) in both control and experimental test tubes. Tubes were incubated at 37 °C for 01 min. A 1 ml of acetic acid was added to stop the reaction in both the tubes. A 200 µl of plasma was added in control tubes and dye was extracted in 4 ml of n-butanol. Test tubes were centrifuged at 3000 x g for 10 min. Absorbance was measured at 560 nm against blank as n-butanol. The activity was expressed in U/mg protein.

3.3.7.4 Paraoxonase Assay

Activity of Paraoxonase enzyme was assayed using the protocol described by Charlton-Menys et al 2006. Initial rate of hydrolysis of paraoxon (O,O-diethyl –O-p-nitrophenule phosphate ) were determined by measuring the liberated p-nitrophenol. The basal assay mixture contained 2.0 mmol/l paraoxon and 2.0 mmol/l of calcium chloride (CaCl₂) in 0.1 mol/l Tris HCl buffer, pH 8.0. A 10 µl of serum was added to the 200 µl of above mentioned mixture. Kinetic analysis was done by giving lag time of 30 min, during this
time interval the enzyme get activated. For basal activity estimation use of sodium chloride in any reagent was devoid. Readings were taken at 405 nm on 1 min, 2 min, 3 min time interval. The standard curve was made by the serial dilution of p-nitrophenol. Enzymatic activity was determined spectrophotometrically in 96-well microtiter plate by microtiter plate reader; Molecular device using software ‘Soft Max Pro’. The activity was expressed in nmol/min/ml.

3.3.8 Antioxidant Status: Markers

3.3.8.1 FRAP Assay

The total antioxidant potential of blood sera was determined using a ferric reducing ability (FRAP) assay described by Benzin and Strain 1996. The FRAP ‘antioxidant power’ measures the change in absorbance owing to the formation of a blue colored Fe\textsuperscript{II} tripyridyltriazine compound from colorless oxidized Fe\textsuperscript{III} form by the action of electron donating antioxidants. The working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/L acetate buffer, pH 3.6, with 1 volume of 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L hydrochloride acid and with 1 volume of 20 mmol/L ferric chloride. Freshly prepared FRAP reagent (1.5 mL) was incubated at 37 °C and a reagent blank reading was taken at 593 nm. Subsequently, 50 µL of sample and 150 µL of deionized water was added to the FRAP reagent. Final dilution of sample in reaction mixture was 1:34. The sample was incubated at 37 °C throughout the monitoring period. The change in absorbance between the final reading selected (4-min reading). Aqueous solutions of known Fe\textsuperscript{II} concentration, in range of 100–1000 µmol/L (FeSO\textsubscript{4},7H\textsubscript{2}O) were used for calibration. All solutions were used on the day of preparation. In the FRAP assay the antioxidant efficiency of the sera under the test was calculated with reference to the reaction signal given by a Fe\textsuperscript{II} solution of known concentration, this representing a one-electron exchange reaction. The absorbance was noted at 593 nm and FRAP potential was expressed in µmoles/l.

3.3.8.2 Thiol Assay

Thiol content of plasma was estimated by the protocol described by Mallikarjunappa and Prakash 2007. A 100 µl of sera was mixed to the 20 µl of 10 mM DTNB in 0.2M Na\textsubscript{2}HPO\textsubscript{4} and incubated at 37 °C with 900 µl of 0.2 M Na\textsubscript{2}HPO\textsubscript{4} containing 2 mM of
Na$_2$EDTA. The solution was mixed in a vortex mixer and incubated for 5 min. The absorbance of supernatant was taken at 412 nm. The absorbance of sample and reagent blank were subtracted from absorbance of sera to reduce the bias. The standard curve was drawn using glutathione in phosphate buffered saline and Total thiol content in sera was expressed in µmoles/l.

3.3.9 Advanced Oxidized End Product: Markers

3.3.9.1 AGE Assay

AGEs were measured by slightly modified protocol described by Mashiba et al 1992. The precipitated plasma proteins were taken and washed with distilled water using 0.2 kDa Amicon ultra filtration tube concentrating membrane (8000 x g at 4 ºC for 20 min) the final concentration was reconstituted to 6 mg/dl by distilled water. NBT reagent was prepared by dissolving 104.28 mg NBT in 500 ml Carbonate buffer (0.1M pH 10.35). A 50 µl these processed sample was taken and in cleaned and dried test tube. Then 200 µl of NBT reagent added and incubated at 37º temperature for 2 hours. Then the volume was make-up up to 2 ml with distilled water, mixed well and measured the absorbance at 550 nm. The NBT reagent was used as blank. Concentration was estimated using plot of standard ketosamine and expressed in nmoles/mg plasma proteins.

3.3.9.2 AOPP Assay

AOPP were measured by spectrophotometry based protocol described by Witko-Sarsat et al 1996. The assay was performed on a microtiter plate. In test wells, 200 ml of plasma diluted 1/5 in PBS was placed on a 96-well microtiter plate, and 20 ml of acetic acid was added. In standard wells, 10 ml of 1.16 M potassium iodide was added to 200 ml of Chloramine-T solution (0–100 mmol/liter) followed by 20 ml of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader against a blank containing 200 ml of PBS, 10 ml of potassium iodide, and 20 ml of acetic acid. The Chloramine-T absorbance at 340 nm being linear within the range of 0 to 100 mmol/liter, AOPP concentrations were expressed as micromoles per liter of Chloramine-T equivalents.
3.3.9.3 Lipid Hydroperoxide: FOX2 Assay
Lipid hydroperoxides (ROOH) were determined by modified FOX2 assay described by Prakash et al. 2004. The FOX2 assay measures hydroperoxides in plasma through ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine (TPP). The FOX2 reagent was prepared, mixing two stock solutions A and B. Stock solution A was prepared by dissolving ammonium ferrous sulphate in 250 mM H₂SO₄, making final concentration 98 mg/dl. Subsequently Xylenol Orange (76 mg) was added to the ammonium ferrous sulphate solution and the mixture stirred for 10 min at room temperature. Solution B was prepared by dissolving 970 mg BHT in 900 ml methanol (HPLC-grade). A working FOX2 reagent was prepared by mixing one volume of solution A with nine volumes of solution B. Final FOX2-reagent comprised Xylenol Orange (100 μmol/L), BHT (4.4 mmol/l), sulphuric acid (25 mmol/l) in 90% (vol/vol) methanol. The extinction coefficient of the FOX2 reagent at 560 nm was routinely checked in solutions of known concentrations of hydrogen peroxide (H₂O₂). Aliquots (180 μl) of sera were transferred into eight centrifuge vials (8 ml). Than 20 μL of 10 mmol TPP in methanol was added to four of the vials to reduce ROOHs, thereby generating a quadruplicate of blanks. Methanol (20 μl) was added to the remaining four vials to produce a quadruplicate of test samples. All vials were then vortexed and incubated at room temperature for 30 min prior to the addition of 1800 μl of FOX2 reagent. After mixing, the samples were incubated at room temperature for another 30 min. The vials were centrifuged at 8000 x g for 10 min. Absorbance of supernatant was measured at 560 nm. ROOH concentration in sera was expressed in μ mol/l.

3.3.9.4 Advanced-oxidized Lipid End-products (ALEs) Assay
Advanced-oxidized Lipid Products was estimated by thiobarbituric acid reaction based method, described by Buege and Aust 1978, concerns the spectrophotometric measurement of the pink pigment produced through reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) and other secondary lipid peroxidation products. The evaluation of absorbance at 532 nm gives a measure of the extent of lipid degradation. A 500 μL of sera was added to a reaction mixture 1 mL formed by equal parts of 15% trichloracetic acid, 0.25 N HCl, and 0.375% thiobarbituric acid, followed by 30 min heating at 95 °C. After cooling either incubation, chromogen was extracted with n-
butanol and read spectrophotometricaly at 532 nm against a reaction mixture »blank« lacking sera but subjected to the entire procedure and extracted with n-butanol. To correct for background absorption, absorbance values at 572 nm were subtracted from those at 532 nm, the latter representing the absorption maximum of the 2:1 TBA:MDA adduct. A molar extinction coefficient of 156000 was used. The level is expressed in terms of malondialdehyde. All determinations were performed in duplicate. The ALEs content was expressed in µmol/L.

3.3.10 Markers of Renal Deterioration

3.3.10.1 GAG Assay
Glycosaminoglycans were estimated using the protocol described by De Jong et al 1989. DMB solution was prepared by dissolved 10.7 mg of DMB dye in one liter formic acid (55 mmol/L, pH 3.3). A 250 µl urine sample was taken in cleaned and dried test tube. Then 2.50 ml Dimethyl methylene blue solution was added and mixed thoroughly. The optical density was measured at 520 nm. The reagent was used as blank, and Heparan sulphate was used as standard solution for the estimation. Glycosaminoglycans were expressed as GAG to creatinine ratio in µg/g creatinine.

3.3.10.2 Sialic Acid Assay
Total Sialic acid content was estimated method described by Skoza and Mahas 1976. A 100 µl of sera was mixed with 800 µl saline and incubated with 100 µl 1N H₂SO₄ at 80°C for 1 hour. Proteins were precipitated by adding 1 ml of 10 % TCA and centrifuged. 100 µl of supernatant was mixed with 400 µl distilled water and successively 250 µl of periodic acid, and incubated at 37°C for 30 min. Periodic acid (0.25M) was prepared by dissolved 1.14 gm Periodic acid in 50 ml 0.125 N H₂SO₄. A 200 µl Sodium Arsenite was added to decolorize the supernatant, and mixed with 500 µl TBA (Thiobarbituric acid). Sodium Arsenite was prepared by dissolved 2 gm in 100 ml 0.5 N HCL. Reaction contents were incubated for 10 minutes in boiling water bath, cooled, mixed with 1.5 ml DMSO (Dimethyl sulphoxide) and colour intensity was read at 549 nm. Sialic acid concentration was expressed as mg/dl using N-Acetyl Neuraminic Acid (NANA) as standard. Sialic acid content was expressed in sialic acid to creatinine ratio in µg/g creatinine.
3.3.10.3 Proteins/peptides Bound Hydroxy-proline
Proteins/peptides bound Hydroxy-proline was measured by the modified alkaline hydrolysis method of Siddiqi and Alhomida 2002. The urinary proteins/peptides were isolated from urine by filtering 0.22 µm filter to remove debris followed by Amicon ultra filtration unit’s cutoff value 0.2 kDa to remove salts. Retained sample washed with deionised distilled water for that centrifuged 8000 x g at 4 °C for 30 min. A 10 µg sample was added to 2 ml NaOH (2 N final concentration) and the mixture was then hydrolyzed by heating in a boiling-water bath for 3-4 hour, volume was adjusted to 2 ml and 100 µl sample was taken then, 900 µl of 56 mm Chloramine T reagent was added to the hydrolyzed sample and oxidation was allowed to proceed at room temperature for 25 min. Then, 1000 µl of 1 m of p-dimethylaminobenzaldehyde (Ehrlich’s reagent) was added to the oxidised sample and the chromophore was developed by incubating the samples at 65 °C for 20 min. The absorbance was read at 550 nm and hydroxyproline concentration in the samples was calculated from the standard curve. Proteins/peptides bound Hydroxyproline was expressed in ng/g creatinine.

3.3.11 GFR Measurement
GFR was measured using formula given by Modified Diet in Renal Diseases equation (MDRD equation) adjusted for Indian population [Singh et al 2009].

\[ \text{GFR}_{\text{MDRD}} (\text{ml/min/1.73m}^2) = 186*(\text{serum creatinine})^{-1.154}*(\text{age})^{-0.203}(0.742 \text{ if female}) \]

3.4 Statistical Analysis
At the onset of study, data was collected from previous diabetes management records. Data were recorded continuously during follow-up visits of the patients to the OPD, using standardized case record forms, as shown in figure; these forms were computerized into an access excel files. The data were closed for analysis on 31 December 2010. The sample size for each analysis was decided on the basis of disease prevalence in population and all the sample size shown statistical power ≥ 80 %. Data was presented as mean and standard deviation (SD) with a normal distribution, and as median with 95th percentile on skewed distribution. Continuous variables were compared using appropriate non-parametric tests. Quantitative variables were compared using paired t test or \( \chi^2 \) test.
A one way ANOVA was used for the analysis of variance followed by LSD and Duncan’s test. Those parameters which compared and did not have a normal distribution, nonparametric tests (Mann Whitney U and the Wilcoxon) were used to observe statistical significance between the groups. Univariate/ multivariate logistic regression, Receiver-operating-characteristic (ROC) curve and other statistical tools were used to analyze the data, described in respective sections. The consistency in results were considered if p < 0.05. All analysis was carried out by using statistical softwares i.e SPSS 17, Sigma STAT and Med Calc.