3. MATERIALS AND METHODS:

3.1 Selection of area:

Nellore and Prakasam districts belong to the coastal area of south India in Andhra Pradesh state. Geographically it is located along the Bay of Bengal. Various population reside in this area with different life styles. This diversity in their life style developed the disorders in specific communities. Settiyaar (Vysya) is a group of population specifically working with high commercial businesses, prone to obesity and becoming diabetic. The present study was conducted on the Settiyaar community who become more susceptible towards diabetic nephropathy.

3.2 Selection of samples:

Eight hundred and twenty (n=820) type II diabetic subjects from 200 families of Settiyaar (Vysya) community in Nellore and Prakasam districts of Andhra Pradesh State were chosen randomly for the present study. A door to door survey with face-to-face interviews were carried out in the same community group to find out the known diabetic (KD) and newly diagnosed diabetic (NDD) subjects. The information collected was entered on a pre-coded questionnaire. Health complaints related to family history, including gastrointestinal complaints, were recorded. Among the total number of samples 200 were KD subjects and remaining 620 were NDD.
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The present study was constructed to analyze the samples that have the renal disorders with the association of diabetes. Further to isolate the selected samples of interest a questionnaire was been prepared and distributed to the selected population. People suffering with regular renal failure with diabetes and newly diagnosed diabetic were separated.

3.2.1 Subjects:

This study was conducted on around 200 families, who were suffering with renal problems associated with diabetes with KD and NDD. They were provided with explanations for all experimental procedures and informed consent was obtained before the beginning of the study. Blood and urine samples were collected from the subjects and preceded for further hematological and biochemical analysis.

3.2.2 Control subjects:

To compare each and every component or biological parameters, a group of normal healthy individuals (n=200) were chosen from the same areas, who are not suffering with any of these disorders. Biological parameters were compared to standard clinical parameters.

3.3 Measurement of physical parameters:

From the isolated test samples (People suffering with renal problems including KD and NDD) physical parameters like height, weight, waist, hip ratio, hypertension and other details were taken at the time of blood and urine sample collection.
3.4 Hematological findings:

An early morning visit was made on a mutually agreed date. Physical details were recorded and blood samples were drawn by venipuncture from each member, and placed in a heparinized tube. Only those who volunteered were included. 5 ml intravenous blood was drawn from each individual in resting position after overnight fasting in heparin contained vacutainers. The whole blood was transported at 4°C to the laboratory where all the analyses were conducted on the day of blood extraction. The hematological parameters were estimated on a Coulter Counter ZF6 with a hemoglobinometer attachment. Hemoglobin (Hb) was determined spectrophotometrically (540 nm) using cyanomethemoglobin method and expressed as g/100ml. Red blood cell counts (RBC) were made in Neubauer chamber with Hayem dilution solution. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Ranzani - Paiva (1991). The red blood cells were separated from the plasma by centrifugation and washed twice with cold physiological saline. Complete blood picture (CBP) was got through the Semi auto analyzer (Model CHEM 400), Electronics India, India.

3.4.1 Estimation of random blood glucose:

Consecutive subjects attending a general practice of random sugar estimation were asked to provide their consent to take part in this present study exploring their estimations of their blood glucose. Following their
consent, they were asked to complete a short questionnaire before
drawing the blood and their blood glucose measured routinely using an
‘OneTouch Ultra’ blood glucose meter. Regular calibration and quality
control were carried out through samples sent to the local hospital
biochemistry laboratory. Normal random blood sugar level should be less
than 200mg/dl.

3.4.2 Estimation of microalbuminuria:

Microalbumin concentration was measured in a fasting urine
sample using an immunoturbidometric assay (Hitachi 902 autoanalyser;
using a kit obtained from Guilin immunetech co. ltd. China. The mean
inter- and intra-assay coefficients of variation were 3.5 and 4.2%,
respectively.

3.4.3 Estimation of serum creatinine:

Blood was collected in to the sterile micro tubes and set at room
temperature to clot. Centrifuge the tube at 3000 rpm for separation of
blood clot with the serum. The supernatant was collected and analyzed
for the presence of creatinine. Serum creatinine levels were all assayed
with the rate- Jaffe reaction on a Hitachi 747 autoanalyzer (Roche
Diagnostics Corp., Indianapolis, Indiana). This assay was calibrated daily
with a Cfas calibrator by using the uncompensated method during the
study period. Normal range of adult males is 0.8 - 1.4 mg/dl: values are
slightly higher in males due to larger muscle mass. In case of adult
females, 0.6 - 1.1 mg/dl: creatinine clearance is increased in pregnancy,
resulting in lower serum levels. In children, 0.2 - 1.0 mg/dl: slight increases with age because values are proportional to body mass.

3.4.4 Collection of blood for lipid profile:

1ml of heparin solution was added to 9ml of normal saline. Approximately 0.25ml of this solution was taken in a disposable syringe and blood was collected in the morning after overnight fasting from a peripheral vein in the same syringe under aseptic precautions to make it to 5ml. The sample was kept at room temperature for 15 minutes and then centrifuged at 3000rpm for 30 minutes. Plasma was separated and kept in plastic vials at -70°C in the biochemistry department till tested. Triglycerides were estimated according to the method of Buckley et al. (1966). Total cholesterol was estimated in plasma by the method described by Annino and Giese (1976). HDL, LDL, and VLDL were isolated using the method of Kostner (1976) and Lopes et al. (1977). Normal values of triglycerides is<150 mgs/dl, total cholesterol is up to <200 mgs/dl, for HDL 30-60 mgs/dl, LDL is 100-190 mgs/dl, VLDL is 20-40 mgs/dl.

3.4.5 Evaluation of thyroid function tests:

Blood specimens were collected from 820 (408 KD and 412 NDD) type II diabetic patients who were enrolled in the study. Of these 5ml of venous blood drawn from each subject, and the blood sample was discharged into a plain sample bottle and allowed to clot. Serum was separated from cells, divided it into three aliquots and stored them frozen
and thawed only when required for use. TSH, T4, FT3 and TT3 were estimated by enzyme immunoassay (EIA) kit method using commercial kits provided by Calbiotech Inc, USA. We adhered strictly to the manufacturer instructions on the procedures. All analysis was done in duplicate and the average of the duplicate data used for calculations.

3.4.6 Evaluation of liver function tests (LFT):

Fasting blood samples were taken from the control and problematic subjects. Part of each serum sample was used for the usual tests performed by the preventive medicine center, and the remainder was conserved at -20°C until further assay related to the current study.

3.4.7 Estimation of serum bilirubin:

Bilirubin is an endogenous anion derived from hemoglobin degradation from the RBC. The classification of bilirubin into direct and indirect bilirubin are based on the original Van der Bergh method of measuring bilirubin. Bilirubin is altered by exposure to light so serum and plasma samples must be kept in dark before measurements are made. Serum levels of total and conjugated bilirubin were measured by Jendrassik and Grof’s (1938) method and serum albumin level was measured by Rodkey’s (1965) method.

3.4.8 Total bilirubin:

This is measured as the amount, which reacts in 30 minutes after addition of alcohol. Normal range is 0.3-1.3 mg/dl (2-15 μmol/L). It is slightly higher by 3-4 μmol/L in males as compared to females. It is this
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factor, which helps to diagnose Gilbert syndrome in males quite easily.

The diazo method of bilirubin estimation is not very accurate especially in detecting low levels of bilirubin.

3.4.9 Direct Bilirubin:

This is the water-soluble fraction. This is measured by the reaction with diazotized sulfanilic acid in 1 minute and this gives estimation of conjugated bilirubin. Normal range 0.3 mg/dl (5.1 μmol/ L)

Indirect bilirubin: This fraction is calculated by the difference of the total and direct bilirubin and is a measure of unconjugated fraction of bilirubin.

3.4.10 Estimation of hepatic marker enzymes:

The aminotransferases (formerly transaminases) are the most frequently utilized and specific indicators of hepatocellular necrosis. These enzymes- aspartate aminotransferase (AST, formerly serum glutamate oxaloacetic transaminase-SGOT) and alanine amino transferase (ALT, formerly serum glutamic pyruvate transaminase-SGPT) catalyze the transfer of the amino group of aspartate and alanine respectively to the keto group of ketoglutaric acid. ALT is primarily localized in the liver but the AST is present in a wide variety of tissues. Serum alkaline phosphatase activity was measured using the method of Bessey et al. (1946), serum ALT activity was measured using the method of Henry et al. (1960). All of the methods described were adapted for semiautomated analysis (Model CHEM 400), Electronics India, India. Serum AST activity was measured by a technique derived from the
technique of the Société Française de Biologie Clinique (Mathieu et al., 1982). Enzyme activity values are expressed as IU/L at 30°C.

3.4.11 Estimation of serum proteins:

Albumin is quantitatively the most important protein in plasma synthesized by the liver and is a useful indicator of hepatic function. Normal serum values range from 3.5 g/dl to 4.5 g/dl. The average adult has approximately 300 to 500 g of albumin. The serum levels of albumin and globulin at any time reflect its rate of synthesis, degradation and volume of distribution (Robinson et al., 1938). The most widely used method of measuring serum protein is the biuret reaction. The principle of this reaction is that serum proteins react with copper sulfate in sodium hydroxide to form a violet "biuret" complex. The intensity of the violet color is proportional to the concentration of protein.

Albumin is generally measured by a dye-binding technique that utilizes the ability of albumin to form a stable complex with bromocresol green dye. The BCG-albumin complex absorbs light at a different wavelength from the unbound dye. This method may overestimate albumin by binding to other proteins. The total globulin fraction is generally determined by subtracting the albumin from the total protein.

3.4.12 Estimation of electrolytes:

Electrolytes are positively and negatively charged molecules called ions that are found within the body cells and extracellular fluids, including blood plasma. A test for electrolytes includes the measurement of sodium,
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potassium etc. Determination of electrolytes in serum was made in the normal and selected subjects. All analyses were done in duplicate. The determination of sodium and potassium was done by an internal standard flame photometer (Berry et al., 1946). Electrolyte concentrations are similar whether measured in serum or plasma. Normal serum sodium concentration is 135–145 mmol/l; in case of serum potassium is 3.6–5.4 mmol/l.

3.4.13 Estimation of glomerular and tubular markers:

3.4.13.1 Estimation of transferrin:

The serum was obtained from healthy donor. In previous investigations, the sample was selected from plasma, but to exclude the various coagulation factors of plasma, serum was taken as origin sample. Furthermore, another advantage of using serum was its lack of any anti-coagulating factors such as chelators which may interact with ferric ion and limit iron saturation. Since, the amount of transferrin estimated by SRID (Single radio immune diffusion kit, Biogene Co) kit in the first sample was 7.5 g, we used 10.5 mg Fe and 11.44 mg anion bicarbonate (7 μL of 0.1 M or 54 μg NaHCO₃ and 7 μL of 0.1 M or 153.16 μg ferric citrate in pH=8, 4°C, 1 h).

3.4.13.2 Estimation of Ig G:

Serum was separated and stored at 4°C till further use. The serum and urinary IgG were measured by sandwich ELISA (Enzyme Linked Immunosorbent Assay) within one week of storage using reagents
supplied by Bangalore Genei, India. The 96 wells ELISA plate (Tarsons) were coated with 100μl of capture antibody (goat anti human IgG, 10μg/ml) in carbonate buffer (50 mM pH 9.6). The plates were incubated at 4°C under humid conditions over night. The wells were aspirated out and washed thrice with PBST (Phosphate buffer saline tween - 20 - 0.1 M pH 7.2). The vacant sites were blocked with 100ml of blocking solution (3% Bovine Serum Albumin in PBST), incubated at room temperature for 1 hour and washed thrice with PBST. 100μl of samples and standards were added to wells in conjugate diluent (0.1% BSA in PBST). The plates were incubated at 37°C for 1 hour, and washed thrice with PBST. Goat anti human IgGHRP antibody with 1:1000 dilution in PBST was added @100μg/well and incubated at room temperature for 1 hour. The plates were washed four times with PBST, 100μl of substrate TMB (tetramethylene blue/ H₂O₂ was added and the reaction was stopped with 50μl/well 1N sulphuric acid after 5 minutes and the O.D. was read at 450 nm in ELISA reader (SpectraMax 190 Absorbance Microplate Reader, Molecular Devices Ltd, USA.). The urine samples were centrifuged in refrigerated centrifuge at 1500 r.p.m. to remove cell debris and stored at -20°C with sodium azide (0.2%) as preservative. Further analysis was made as earlier.

3.4.13.3 Estimation of antitrypsin:

Oxidative antitrypsin (AT) was analyzed using an ELISA with a monoclonal antibody against oxidized α₁-AT in which chloramine T-
oxidized α1-AT was the antigen (Ueda et al., 2002). The sensitivity of measurement was 1.0 ng/ml with an inter CV of <6.7%.

3.4.13.4 β2 –microglobulin assay:

Under aseptic precautions venous blood was drawn and serum separated. The samples were frozen at -70° C until assay. The serum was analyzed by Enzyme linked immunosorbent assay (β2–macroglobulin EIA kit, Immunotech, France). 2.4mg/ L was used as the upper limit, when 97% of normal values are below this cut off value.

3.5 Serum and urine ACE Level Measurement:

Serum or urine ACE level was measured by a colorimetric method (colorimetric assay kit, Fujizoki Assay, Tokyo, Japan) using p-hydroxyhippuryl-L-histidyl-L-leucine as the substrate (Kasahara and Ashihara, 1981).

3.5.1 Determination of ACE Genotypes:

The D and I alleles were identified on the basis of polymerase chain reaction (PCR) amplification of the respective fragments from intron 16 of the ACE gene and size fractionation and visualization by electrophoresis. DNA was extracted from peripheral leukocytes with standard techniques. PCR was performed with 20 pmoles of each primer: sense oligo 5’CTGGAGACCATCCCATCCTTTCT3’ and anti-sense oligo: 5’GATGTGGCCATCACATTCGTCAGAT3’ in a final volume of 25 µl, containing 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.2mM of each dNTP, and 1.25 unit of Taq polymerase (Perkin Elmer-Cetus,
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Norwalk, CT). The DNA was amplified for 30 cycles with denaturation at 94° C for 30 s, annealing at 58° C for 30 s and extension at 72° C for 1 min, followed by final extension at 72° C for 5 min (DNA Thermal Cycler 480, Perkin Elmer-Cetus) (Saiki et al., 1988; Rigat et al., 1992). PCR products were electrophoresed in 2% agarose-gel with 5 µg ethidium bromide per milliliter. The amplification products of the D and I alleles were identified by 300-nm ultraviolet trans-illumination as distinct bands (D allele: 191 bp; I allele: 478 bp) Because the D allele in heterozygous samples is preferentially amplified, each sample found to have the DD genotype was subjected to a second independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (hace 5a, 5'TGGGACCACAGCGCCCGACTAC3';hace 5c, 5' TCGCCAGCCCTCCCA TGCCCATAA3'), with identical PCR conditions except for an annealing temperature of 67 °C. The reaction yields a 335-bp amplicon only in the presence of an I allele, and no product in samples homozygous for DD (Shanmugam et al., 1993; Lindpaintner et al., 1995).

3.6 Statistical Analysis:

Statistical analysis was carried out using SPSS for windows 10.0 software (SPSS Inc., Chicago, IL, USA) and Microsoft Excel. Values were reported as mean ± standard deviation. SD was not more than 10%. The difference between groups was compared by Pairwise Multiple Comparison Procedures. p value <0.001 was considered statistically significant.