II. MATERIALS AND METHODS

The present study includes the various risk factors and different epidemiological conditions on Diabetes mellitus in rural agrarian population of 37 villages, viz. Kattuputhoor, Kadukkarai, Kurathiyarai, Vilankadu, Kesavanputhoor, Azhagiapandiapuram, Therisanamcope, Thuvarankadu, Veeravanallur, Arumanallur, Boothapandi, Thazhakudy, Navalkadu, Esanthimangalam, Manathittai, Erachakulam, Veeranarayananamangalam, Melaputheri, Kezhaputheri, Thirupathysaram, Vellamadam, Thovazhai, Aralvaimozhi, Puthukiramam, Kulasekaranputhoor, Karuppukottai, Theroor, Marungoor, Eraviputhoor, Kariamanickapuram, Asramam, Kakkumoor, Kurichi, Parakkai, Ozharavilai, Kottaram and Thamaraikulam (North). The study areas are located in the eastern part of Kanyakumari district, Tamil Nadu, the southern end of the Indian sub-continent.

Study area

Kanyakumari district lies between $77^\circ 15'$ of the eastern longitudes and $8^\circ 03'$ and $8^\circ 35'$ of the northern latitudes. It is a smallest district in Tamil Nadu with a land spread of 1,684 km$^2$ with forests, wetlands, fresh water and marine resources. The district is endowed by nature with several hills and mountains. It is with diverse locality factors and having an elevation from sea level to 1829 m.

The study district is well known for various agrarian goods. The district once called “the granary of Travancore” is fertile with
hundreds of water bodies, and canal irrigation systems. Rubber
and spice plantations are found on the hilly terrain, while paddy
fields, plantain and coconut plantations are found on the plains.
Rice is one of the major crops in 400 km² crop area. Rice is the
staple food and for some people, in the hilly areas tapioca is the
staple food.

The district is generally hilly and plains near the coast. The
land from the sea coast gradually rises from sea level to the
Western Ghats hills in the deep interior of the district. The
southern boundary (coastal) is the Gulf of Mannar (Bay of Bengal)
while on the south and the south west, the boundaries are the
Indian Ocean and the Arabian Sea. The district has 62 km of coast
on the western side (Arabian Sea coast) and 6 km. of coast on the
southern side (Bay of Bengal). The district population is
16,76,034 (2001 census) which includes 8,32,269 males and
8,43,765 females spreading in an area of 1684 km². The total
literacy rate of the district is 78 percent, and the male literacy rate
is 80 percent, where as in females it is 76 percent.

Climate

Generally a sub-tropical climate prevails in the district
through out each calendar year, which includes summer, south
west monsoon and north east monsoon seasons. The maximum
and the minimum mean temperature for the study periods 2008,
09 and 10 are: 31.07°C and 24.01°C; 31.49°C and 24.66°C;
31.62°C and 24.1°C respectively. The average annual rain fall for
the years 2008, 09 and 10 are: 144mm; 120mm; 160mm
respectively. The mean humidity values recorded in the district during the periods 2008, 09 and 10 are: 80.32% at 8.30 hr, 84.07% at 17.30 hr; 80.67% at 8.30 hr, 84.3% at 17.30 hr; 80.8% at 8.30 hr, 84.9% at 17.30 hr respectively.

**Study population**

The study subjects Nanjil Nattu Vellala, an agrarian, endogamous, native, non-vegetarian, forward caste, Hindu people settled/dispersed from the coast to the mountain (Western Ghats) bases of the south eastern, eastern and north eastern parts of Kanyakumari District. Nanjil Nadu, is the present Agastheeswaram and Thovazhai Taluks (administrative sub divisions) of the district. The word “Vellalan” is derived from “vellam” (water) and “anmai” (management), meaning cultivation (or) tillage.

The study covered a sizable number of population (i.e) 5000 subjects, which includes 2561 (51.22%) males and 2439 (48.78%) females of age between 19 – 75 years. The survey population is with different socio-economic capabilities with agricultural back ground. The sample population was interviewed in 37 villages, and the distance between the near by coastal study village (Thamaraikulam – North) and Kattuputhoor (high range) is approximately more than 40 km (on the road). In each village a minimum of 20 percent (or) around 20 percent of the permanent settlers were interviewed for the present study. The survey group is residing in equal number of modern houses, old as well as small tiled and thatched houses.
**Diabetes diagnosis**

To assess the magnitude of the disease, house to house survey is performed with a well designed questionnaire. The present study is carried out during the period 2008-10 and is restricted to rural, agrarian areas. Fig. .9. and .9(a). shows the site map of the study areas. Random selection of houses in the survey areas are selected. The survey is made without any bias of occupation or social class. Persons of all age group including men, women and adolescents staying in the same household are screened for diabetes with or without clinical manifestations by making a door to door visit. This procedure eliminated selection with respect to age and sex as every person available in the household got an equal chance of being examined. The clinical problems of diabetes, are screened by experienced medical practitioners.

The major objectives are to discuss the impacts of sex, age, socio-economic status, food preference, BMI, waist-hip ratio, hypertension, blood groups, rate of incidence, habituation, food habits, age of onset, life style, biomarkers, etc. on diabetes. This community based study will try to create an awareness and it will try to unlock the unknown facts about diabetes among the people in the study areas. The present study will try to find out the reasons if any, for the shift from higher to lower age groups, affluent to ordinary people and idle people to active skilled labourers. Suitable hypotheses will be made based on the findings.
and the validity of the data will be analysed by proper statistical methods.

Standard methods are used to screen the patients and for various chemical, biochemical, enzyme analysis and so on.

1. Detection / Screening of diabetics

During the study period (i.e) January 2008 to December 2010, 5000 people from 37 villages in a spread of approximately more than 40 km on the road were screened for diabetes. The study population includes 2561 males and 2439 females within the age 19 – 75 yrs, who have settled near the coastal region, plain and high range.

Detection of diabetics has been performed by Glucocare meter. Glucocare is used worldwide. It defines the degree of discolouration and converts into a glucose concentration value. It is designed only for use with Glucocare Test Strip. The meter will give a “LO” sign at too low values (under 2.2 mmol/L and a “Hi” sign at too high values (above 25 mmol/L). Photometric accuracy is better than ±1 percent.

2. Blood grouping and cross matching

To identify the different blood groups and analyse the cross matching slide method is used.

3. Counting of blood cells

By haemocytometric method, with the RBC and WBC diluting fluids, total erythrocytes and total leucocytes can be found out by
the specific procedures. Normal range: WBC: 4-10,000 cells/cu. mm, RBC: 4.2 – 6 million/cu. mm.

4. **Measurement of haematocrit value or packed cell volume**

(PCV)\(^{235}\)

RBC’s are packed using centrifugal force and the volume is measured. PCV is calculated by Wintrobe’s tube method and the value is expressed in percentage. Normal range: 40 – 50%.

5. **Estimation of haemoglobin (Hb)\(^{236}\)**

For the determination of haemoglobin in whole human blood, Cyanmethaemoglobin method is used. The cyanmethaemoglobin complex formed during the assay is directly proportional to the Hb content of the whole blood, when measured at 540 nm and the values are expressed in g / dL. Reference range: 13 – 17 g /dL.

6. **Counting of platelets (thrombocytes)\(^{235}\)**

For counting of platelets in human blood anticoagulated blood is used. Count the number of platelets in the four corner squares (as for WBC) by the method described. Normal range: 1.5 – 4 lac/cu. mm.

7. **Counting of reticulocytes\(^{235}\)**

For counting of reticulocytes in whole human blood anticoagulated blood is used. Microscopic (oil immersion) examination of reticulocytes has been made by the method described. Reference range: 0.5 – 2.5%.
8. Measurement of erythrocyte sedimentation rate\textsuperscript{235}

Measurement of ESR has been done by the method (Westergren) described. Normal range: 0 – 25 mm/hr.

9. Quantitative determination of total protein in human serum

by modified Biuret, end point assay\textsuperscript{237, 238}

The peptide bonds of proteins react with cupric ions in alkaline solution to form a coloured chelate, the absorbance of which is measured at 578 nm. The biuret reagent contains sodium – potassium tartrate, which helps in maintaining solubility of this complex at alkaline $p_H$. The absorbance of final colour is proportional to the concentration of total protein in the sample and the values are expressed in g / dL. Normal range: 6.4 – 7.8 g/dL.

10. Quantitative determination of serum albumin\textsuperscript{238, 239}

At $p_H$ 3.68, albumin acts as a cation and binds to the anionic dye bromocresol green, forming a green coloured complex. The absorbance of final colour is measured at 630 nm. The colour intensity of the complex is proportional to albumin concentration in the sample, which is expressed as g / dL.

Total serum globulin = total protein – albumin. Reference range: 2.5 – 3.5 g/dL

Conversion factor

Albumin concentration in g/ L =albumin concentration in g / dL x 10. Reference range: 3.5 – 5.2 g/dL.

$A/G$ ratio  = total albumin / total globulin.
11. Quantitative estimation of serum bilirubin (total, direct and indirect)\textsuperscript{240}

Direct bilirubin in the sample reacts with the diazotized sulfanilic acid forming a coloured complex that can be measured by spectrometry. Both direct and indirect bilirubin couple with diazo in the presence of centrimide. The terms “direct” and “total” refer to the reaction characteristics of serum bilirubin in the absence or presence of solubilizing (accelerating) reagents. The direct and indirect bilirubin are only approximately equivalent to the conjugated and unconjugated fractions. For the measurement of indirect bilirubin, subtract direct bilirubin value from the total bilirubin value, and the unit of measurement is mg/dL. Reference range: bilirubin (total): 0 – 1.11 mg/dL; bilirubin (direct): 0 – 0.25 mg/dL; bilirubin (indirect): 0 – 0.85 mg/dL.

12. Quantitative estimation of serum insulin (fasting)\textsuperscript{241}

The ADVIA Centaur Insulin Assay is a two-site sandwich immunoassay using direct chemiluminescent technology which uses constant amounts of two antibodies. The first antibody, in the Lite Reagent, is a monoclonal mouse antinsulin antibody labelled with acridinium ester. The second antibody, in the Solid Phase, is a monoclonal mouse anti-insulin antibody, which is covalently coupled to paramagnetic particles. The amount of insulin detected by this method is expressed in µIU/ml. Normal range: 2.6 – 37.6 µIU/ml.
13. **Quantitative determination of HbA$_{1C}$ by High Performance Liquid Chromatography (HPLC)**$^{242, 243}$

After preparing the hemolysate, where the labile fraction is eliminated, haemoglobins are retained by a cationic exchange resin. Haemoglobin A$_{1C}$ (HbA$_{1C}$) is specifically eluted after washing away the haemoglobin A$_{1a + b}$ fraction and is quantified by direct photometric reading at 415 nm and the values are expressed in percentage. Reference range: Normal: 4.4 – 67%; Excellent control: 6.7 – 7.3%; Good control: 7.3 – 9.1%; Poor Control: > 9.1%.

14. **Measurement of serum cholesterol level in human serum by CHOD – PAP Method**$^{244, 245}$

Estimation of cholesterol involves various enzyme catalysed reactions. Absorbance of quinoneimine so formed is directly proportional to cholesterol concentration in the specimen and the values are expressed in mg/dL. Reference range: 140 – 250 mg/dL.

15. **Quantitative determination of High Density Lipoprotein Cholesterol (HDL-C) in human serum by Accelerator Selective Detergent Method**$^{246, 247}$

The HDL – C assay is a homogenous method for direct measurement of HDL – C levels in serum without any off-line pretreatment or centrifugation steps. HDL – C values are expressed in mg / dL. Normal range: 40 – 60 mg/dL.

16. **Quantitative estimation of Low Density Lipoprotein Cholesterol (LDL-C) in human serum by Selective Detergent Method**$^{248, 249}$

The LDL – C assay is a homogenous method for direct measurement of LDL – C in serum, without any off-line
pretreatment or centrifugation steps. The LDL-C values are expressed in mg/dL. Normal range: Upto 130 mg/dL.

VLDL cholesterol is calculated from the assay and the values are expressed in mg/dL. Reference range: 0 – 25 mg/dL.

17. **Quantitative measurement of triglycerides by GPO- Trinder Method**\(^{250, 251}\)

The intensity of chromogen (quinoneimine) formed during the assay is proportional to the triglycerides concentration in the sample when measured at 505 nm and the values are expressed in mg/dL. Reference range: 25 – 160 mg/dL.

18. **Estimation of blood urea nitrogen (BUN) by GLDH – Urease method**\(^{252}\)

The estimation of urea in serum involves the catalytic reactions by urease and glutamate dehydrogenase enzymes. The rate of decrease in absorbance is monitored at 340 nm and is directly proportional to urea concentration in the sample which is expressed in mg/dL. Normal range: 5 – 21 mg/dL.

19. **Estimation of serum ferritin by CLIA – Siemons ADVIA Centaur Method**\(^{253}\)

ADVIA Centaur Ferritin assay is a two-site sandwich immunoassay using direct chemiluminescent technology, which uses constant amounts of two anti-ferritin antibodies. First antibody, in the Lite Reagent, is a polyclonal goat anti-ferritin antibody labelled with acridinium ester. The second antibody, in
the Solid Phase, is a monoclonal mouse anti-ferritin antibody, which is covalently coupled to paramagnetic particles. The values are expressed in ng/ml. Normal range: 22 – 322 ng/ml.

20. Estimation of c-peptide by ELISA\textsuperscript{254, 255}

The Bio-line C-peptide – ELISA is a solid phase enzyme amplified sensitive immunoassay performed on a microtitre plate. The amount of substrate turn over is determined colourimetrically by measuring the absorbance, which is inversely proportional to the c-peptide concentration, which is expressed in ng/ml. Normal range: 0.7 – 1.9 ng/ml.

21. Quantitative determination of creatinine in human serum

by Alkaline picrate – Synchron CX5 PRO\textsuperscript{237, 256}

Creatinine reacts with picric acid in an alkaline medium to form an orange coloured complex. The rate of formation of this complex is measured by reading the change in absorbance at 505 nm in a selected interval of time and proportional to the concentration of creatinine. The reaction time and the concentration of picric acid and sodium hydroxide have been optimized to avoid interference from ketoacids. The amount of serum creatinine is expressed in mg/dL. Normal range: 0.66 – 1.11 mg/dL.

22. Quantitative determination of serum glucose by GOD-PAP Method\textsuperscript{257, 258}

Enzymatic determination of serum glucose is based on two enzymes (viz) glucose oxidase (GOD) and peroxidase (POD). The
amount of glucose in the serum samples are measured at 505 nm and expressed in mg/dL. Reference range: Glucose (F): 70 – 110 mg/dL; Glucose (PP): 70 – 140 mg/dL.

23. Estimation of cholinesterase by DTNB method

The rate of absorbance of cholinesterase is determined photometrically at 405 nm and the values are expressed in U/L. Normal range: 5600 – 11200 U/L.

24. Quantitative determination of α-amylase in human serum

by CNPG3 synchron CX5 PRO

α-amylase hydrolyses 2-chloro-p-nitrophenyl –D maltotriose (CNPG3) to release 2 chloro-nitrophenol (CNP), CNPG2, maltotriose, and glucose. The rate of increase in absorbance due to the formation of CNP is measured at 405 nm and is proportional to the α-amylase activity in sample and the values are expressed in IU/L. Reference range: 20 – 80 IU/L.

25. Quantitative determination of creatinine kinase (MB) by immuno-inhibition method

The procedure involves measurement of creatinine kinase activity in the presence of an antibody to creatinine kinase-M monomer. This antibody completely inhibits the activity of CK-MM and half of the activity of CK-MB, while not affecting the B subunit activity of CK-MB and CK-BB. Then use CK method to quantitatively determine CK-B activity. The CK – MB activity is
obtained by multiplying the CK-B activity by two. The unit of measurement of CK-MB activity is U/L. Reference range: upto 25 U/L.

26. **Estimation of lactate dehydrogenase in human serum by**

   **DGKC method**\(^\text{261, 263}\)

   Quantitative determination of LDH on photometric systems and the values are expressed in IU/L. Normal range: 225 – 450 IU/L.

27. **Estimation of alkaline phosphatase activity in human serum by**

   **DGKC method**\(^\text{238}\)

   In alkaline solution ALP catalyses the hydrolysis of p-nitrophenyl phosphate into p-nitrophenol and phosphate. The rate of formation of p-nitrophenol, proportional to the ALP activity, is measured at 405 nm and the values are expressed in IU/L. Normal range: 110 – 380 IU/L.

28. **Estimation of acid phosphatase in serum by end point method (PNPP)**\(^\text{238, 264}\)

   In an acid medium, acid phosphatase hydrolyses paranitrophenyl phosphate (PNPP) to paranitrophenol and phosphate. The intensity of the coloured complex which absorbance is proportional to the acid phosphatase activity is measured at 405 nm and the values are expressed in IU/L. Normal range: upto 9 IU/L.
29. **Estimation of aspartate aminotransferase (AST) in human serum by UV kinetic (IFCC) method**\(^{265}\)

There is a decrease in absorption at 340 nm as NADH is converted to NAD. The rate of decrease in absorbance is measured and is proportional to SGOT activity in the sample and is expressed in IU/L. Reference range: < 40 IU/L.

30. **Estimation of alanine aminotransferase (ALT) in human serum by UV kinetic International Federation of Clinical Chemistry (IFCC) Method**\(^{266, 267}\)

Quantitative determination of SGPT by UV kinetic method and the values are represented in IU/L. Normal range: Upto 31 IU/L.

31. **Estimation of gamma glutamyltransferase (GGT) by gamma glutamyl p-nitroanilide (GGPNA) – Synchron CX5 Pro**\(^{238, 268}\)

The rate of formation of p-nitroaniline is directly proportional to GGT activity in the sample, is measured at 405 nm and the values are expressed in IU/L. Reference range: 1 – 24 IU/L.

32. **Estimation of urine protein or microalbuminuria or proteinuria by pyrogallol red dye binding method**\(^{269, 270}\)

The pyrogallol red is combined with molybdate to form a red complex which turns to blue-purple colour in the presence of protein. The colour intensity is read at 600 nm in a colorimeter and the readings are expressed in mg/dL. Normal range: 0 – 10 mg/dL.
33. **Estimation of uric acid in serum samples by uricase method**

Uricase acts on uric acid to produce allantoin, carbon-dioxide and hydrogen peroxide in the presence of peroxidase reacts with a chromogen (amino-antipyrine and dichloro-hydroxybenzene sulfonate) to yield quinoneimine, a red coloured complex. The absorbance measured at 520 nm is proportional to the amount of uric acid present in the samples, and the values are expressed in mg/dL. Reference range: 2.6 – 6.0 mg/dL.

34. **Quantitative determination of thyroid stimulating hormone (TSH) in serum by CLIA**

The ADVIA Centaur TSH assay is a two-site sandwich immunoassay using chemiluminometric technology, which uses constant amounts of two antibodies. The first antibody, in the Lite Reagent, is a monoclonal mouse anti-TSH antibody labelled with acridinium ester. The second antibody, in the Solid Phase, is a polyclonal sheep anti-TSH antibody which is covalently coupled to paramagnetic particles. The values obtained by this chemiluminometric immuno assay (CLIA) is expressed in µIU/ml. Normal range: 0.35 – 5.5 µIU/ml.

35. **Estimation of free thyroxine (3, 5, 3’ 5’-tetraiodothyronine, L-thyroxine (or) fT4 in serum by Chemiluminometric immunoassay (CLIA)**
The ADVIA Centaur FT4 assay is a competitive immunoassay using direct chemiluminescent technology. FT4 in the sample competes with acridinium ester-labelled T4 in the Lite Reagent for a limited amount of biotinylated polyclonal rabbit anti-T4 antibody. Biotin-labelled anti-T4 is bound to avidin that is covalently coupled to paramagnetic particles in the solid phase. The readings obtained by this CLIA is expressed in ng/dL. Normal range: 4.5–10.9 ng/dL.

36. Quantitative determination of free T3 (or) triiodothyronine in human serum by CLIA²⁷⁴⁻²⁷⁶

The ADVIA Centaur FT3 assay is a competitive immunoassay using direct chemiluminescent technology. FT3 in the sample competes with a T3 analog, which is covalently coupled to paramagnetic particles in the Solid Phase for a limited amount of a combination of acridinium ester – labelled monoclonal mouse anti – T3 antibodies in the Lite Reagent. An inverse relationship exists between the amount of FT3 present in the sample and the amount of relative light units (RLUS) detected by the system and the values are expressed in pg/ml. Reference range: 2.3 – 4.2 pg/ml.

37. Estimation of total thyroxine (TT4) in human serum by CLIA²⁷⁴, ²⁷⁷, ²⁷⁸

The ADVIA Centaur T4 assay is a competitive immunoassay using direct chemiluminescent technology. T4 in the sample competes with a T4 analog, which is covalently coupled to paramagnetic particles in the solid phase, for a limited amount of
acridinium ester-labelled monoclonal mouse anti-T4 antibody in the Lite Reagent. The values are expressed in µg/dL. Normal range: 4.5 – 10.9 µg/dL.

38. **Estimation of total triiodothyronine (TT3) in human serum by CLIA**[^274] ,[^277] ,[^278]

The ADVIA Centaur T3 assay is a competitive immunoassay using direct chemiluminescent technology. T3 in the sample competes with a T3 analog, which is covalently coupled to a paramagnetic particles in the solid phase, for a limited amount of acridinium ester – labelled monoclonal mouse anti-T3 antibody in the Lite Reagent. The values obtained by this method are expressed in ng/dL. Reference range: 0.6 – 1.81 ng/ml.


At mildly acidic pH, metallo-chromogen Arsenazo III combines with calcium to form a coloured complex which absorbance measured at 650 nm is proportional to the amount of calcium in the sample and the values are expressed in mg/dL. Normal range: 8.6 – 10 mg/dL.

40. **Estimation of serum sodium, potassium and chloride by ion-selective electrode method**[^280] ,[^281]

Serum sodium, potassium and chloride are estimated by ion-selective electrodes, and the methods described elsewhere and the values are expressed in mmol/L. Reference range: sodium: 136–146 mmol/L; potassium:3.5–5.0 mmol/L; chloride:98-106 mmol/L.
41. Separation and quantitation of serum protein by agarose gel electrophoresis

Separation and quantitation of serum protein is done by agarose gel electrophoresis.

42. Measurement of body mass index (BMI)

BMI is a gross estimate for the amount of fat in the body. It is calculated by the formula weight in Kg/height in m\(^2\) and the method described elsewhere.

43. Measurement of waist-hip ratio (WHR)

WHR has been used as an indicator or measure of the health of a person, and the risk of developing serious health conditions.

44. Statistical analysis

Statistical analysis are made with SPSS statistical package (version 11).