CHAPTER - 3

Methodology
Study Design

This cross sectional study was carried out by conducting 10 health camps in and around Mysore and Kutta, Kodagu district from June 2011 to January 2012. A pilot study was done to identify the areas in Mysore where most of the Kodavas are residing. Health camps were conducted in various residential areas in Mysore like Vijayanagar 3rd stage, Vijayanagar 1st stage, Hebbal, Hinkal, Gokulam, Siddhartha Layout and J.P Nagar. Campaigning for the health camps was done with the help of area associations and Kodava Samaj, Mysore. Most of the subjects under study were from urban area and educated. Some of the subjects were from rural areas of Kodagu district like Kutta, Virajpet, Kushalnagar and Cherambane. A health camp was conducted in Kutta government hospital, Kutta wherein 81 subjects participated. They were informed about the study and its purpose by pamphlets, through phone calls or by word of mouth by the members of area association. All participants were asked to attend camp in fasting state.

All the participants were asked to sign the informed consent form. The subjects were asked to fill personal details like age, spouse name, family name, maiden family name (for women) which are very essential when we are studying about this community. A standardized questionnaire related to their clinical history, medications/treatment, diet, alcohol consumption, smoking status and physical activity were recorded. This study was approved by Institutional Ethical Committee (IOE) Anthropological Survey of India, Kolkata.
Study Population

All study samples were from Kodavas originally from Kodagu district. They were collected from Mysore district, Karnataka. According to the Karnataka unit of Bureau of economics and statistics in 2011, the population of Kodavas in Mysore was 15,000 and the overall population was 1,25,000.

Inclusion criteria

Age between 25-85 years

Should be from Kodava community

Willingness to give the consent for the study

Exclusion criteria

Subjects whose one of the parents or grandparents was Non-Kodavas.

Subjects below the age of 20

Women who have undergone hormone replacement therapy
Area under study

Mysore and Kodagu map showing the places where the camps were conducted.

Sample number calculation

Sample number was calculated using creative research system survey software with confidence level of 95% and confidence interval of 4.74 with a population of 15,000, which gave a sample size of 418.

Subjects and Methods

This cross sectional study was carried out by conducting 10 health camps in and around Mysore. A total of 426 subjects aged between 25 to 85 years attended the camp. Six subjects were excluded from the study as they were Non Kodavas. Two female subjects belonging to the other community but married to Kodava subjects were excluded from the study. Total of 418 subjects were in the study of which 81 subjects were from rural areas of Kodagu district.
Sample collection

After overnight fast, 10ml of venous blood was drawn in EDTA vacutainers from the subjects by a phlebotomist. All blood samples were stored in ice pack and the plasma was separated using centrifuge at 1000 rpm for 10 minutes.

Anthropometric measurements

Anthropometric measurements like height, weight, waist and hip circumference were recorded for all the subjects. Height was measured using Holtain Anthropometric scale and weight was checked without shoes and with light clothing. Waist circumference (WC) was measured using a flexible inextensible tape placed horizontally at the midpoint between the lowest rib and the iliac crest. Hip circumference (HC) was measured at the widest circumference over the major trochanters with the subject standing erect. Waist hip ratio (WHR) was calculated using these measurements.

Total Body - Fat Percentage, Body Mass Index (BMI) and Basal Metabolic Rate (BMR) was measured using Omron fat monitor with the subject standing erect without shoes.

Blood Pressure measurement

We used the auscultatory method of blood pressure measurement using sphygmomanometer. The participants were allowed to be comfortable in sitting position and two readings were recorded twice at five minutes interval with a cuff on the right arm. The average of two readings were considered and the average blood pressure was classified according to WHO, wherein BP above 130/85 was considered hypertensive. The pulse rate was also noted.
Biochemical Analysis

All the samples were tested for glucose, High Density Lipoprotein-Cholesterol (HDL-C), Low Density Lipoprotein-Cholesterol (LDL-C), Total Cholesterol (TC), Triglyceride (TG), Uric Acid, CRP using ERBA kits in automated biochemical analyzer EM360, Transasia. ApoA1, ApoB, Lp(a) were tested using Randox kits in the automated Biochemical analyzer.

Fasting glucose

All the plasma samples were checked for glucose using Liquixx reagents by Trinder’s method. In this method, glucose is oxidized to yield gluconic acid and hydrogen peroxide in the presence of glucose oxidase. The enzyme catalyzes the oxidative coupling of 4-aminoantipyrine with phenol to yield a colored quinoneimine complex, with absorbance proportional to glucose in the sample.

Low Density Lipoprotein (LDL-C)

All the plasma samples were checked for LDL-C using liquid stable reagent by coupled classic precipitation method using polyvinyl sulfonic acid (PVS) and polyethylene glycol methyl ether (PEGME). In this method LDL reacts with PVS and PEGME which makes them inaccessible and the addition of R2 containing a specific detergent releases LDL from PVS/PEGME complex. The released LDL combines with hydrogen peroxide which is quantified by Trinder reaction.

High Density Lipoprotein (HDL-C)

All the plasma samples were checked for HDL using liquid stable reagent by coupled classic precipitation method using polyvinyl sulfonic acid (PVS) and polyethylene glycol methyl ether (PEGME). They make the LDL, VLDL and
chylomicron inaccessible by cholesterol oxidase and cholesterol esterase, and the enzyme selectively react with HDL to produce H2O2 which is detected through Trinder reaction.

**Triglyceride**

All the plasma samples were checked for Triglycerides using GPO method wherein the triglycerides are lysed using lipase and it releases free acids and glycerol. It is phosphorylated by glycerol kinase and adenosine triphosphate to produce glycerol 3 phosphate and ADP. It is oxidized to Di hydroxyl acetone Phosphate using Glycerol 3 phosphate oxidase and hydrogen peroxide. In the Trinder type color reaction catalyzed by peroxidase, the hydrogen peroxide reacts with 4-aminoantipyrine and TOOS to produce a red colored dye. The absorbance of this dye is proportional to the concentration of triglyceride present in the sample.

**Total Cholesterol**

All the plasma samples were checked for Total cholesterol by CHOD-PAP method. The cholesterol ester is hydrolyzed by cholesterol esterase to cholesterol and fatty acids, It is then oxidized using cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide which combines with 4-aminoantipyrine producing a chromophore which may be quantified at 505nm.

**C Reactive Protein (CRP)**

All the plasma samples were checked for CRP using turbidometric immunoassay. This involves measuring the antigen –antibody reaction by the endpoint method. The samples are mixed with buffer and read at 340nm and then added with antiserum, incubated at room temperature for 5 minutes and OD was measured.
Uric Acid

All the plasma samples were checked for Uric acid using liquid stable reagent by the uricase PAP method based on Trinder’s reaction. The uric acid present in the sample is hydrolyzed by uricase to produce allantoin, and it reacts with peroxidase to form a quinoneimine dye. The intensity of the color formed is proportional to the uric acid concentration.

Lipoprotein (a)

All the plasma samples were checked for Lipoprotein (a) using turbidometric immunoassay by the antigen-antibody reaction by the end-point method. Mix 30µl of diluted samples with 900µl buffer and take the OD at 600nm. Now add 80µl of latex, mix and incubate for 5 minutes and read the OD at 600nm.

Lipoprotein Lipase

All the plasma samples were checked for advanced homogenous micelle technology. The chromogenic substrate is cleaved by the lipase which produces 1,2-O-dilauryl-rac-glycerol and an unstable intermediate, glutaric acid (methylresorufin) ester. This decomposes spontaneously in alkaline solution. The color intensity of red dye is directly proportional to the lipase activity.

Apolipoprotein B

All the plasma samples were checked for Apolipoprotein B using turbidometric immunoassay by the antigen-antibody reaction by the end-point method. In this method 40µl of sample is mixed with 900µl of buffer and optical density is read at 340nm. To this add 80µl of antiserum, mix and incubate for 5 minutes at room temperature and read the OD at 340nm.
Apolipoprotein A1

All the plasma samples were checked for Apolipoprotein A1 using turbidometric immunoassay by the antigen-antibody reaction by the end-point method. In this method, 10µl of sample is mixed with 900µl of buffer and then add the antiserum. Read optical density (OD1) at 340nm, mix and incubate for 5 minutes at room temperature and read Optical density (OD2) of samples, standards and controls at 340nm.

Homocysteine

Principle of Immulite Instrument

It is a automated machine which performs a solid phase, competitive chemilumiscnt enzyme immunoassay. It involves a competitive binding between the labeled antigens and non labeled antigens to the limited amount of antibody binding sites. Along with this a chemilumiscnt substrate is also loaded. The analyser will mix the sample, labeled antigen and antibody together. After this it will add the substrate to chemically react with the enzyme to produce light which is captured and measured. The amount of bound labeled antigen is determined and is inversely proportional to the amount of non labeled antigen. Then the overall concentration of analyte is calculated and generated.

The homocysteine levels were measured using homocysteine kits in Immulite 1000. 15µl of plasma samples were pretreated with 300µl of adenosyl-L-homocysteine hydrolase and dithiothreitol and incubated at 37°C in a water bath or oven for 30 minutes to release S-adenosyl-homocysteine(SAH). The treated sample and alkaline phosphatase labeled anti SAH antibody are simultaneously introduced into a test unit containing an SAH-coated polystyrene bead. During 30 minute
incubation, the converted SAH competes with the immobilized SAH for binding the alkaline phosphatase labeled anti SAH antibody conjugate. Unbound enzyme conjugate is removed by centrifugal wash. Substrate is added which contains the phosphate, photons are emitted by the enzyme-substrate binding and is read by the luminometer.

**CVD risk marker analysis**

**Principle of Luminex**

Luminex is based on luminex Xmap technology. The microspheres are internally coated with two fluorescent dyes and color beads in equal concentration can be created each of which is coated with specific capture antibody. After the analyte in the test sample is captured by the bead biotinylated detection antibody is introduced. Then the reaction mixture is incubated with streptavidin PE conjugate, the reporter molecule. These are passed through laser which excites the internal dyes and then second laser excites the PE. Finally a high speed digital signal processors identify each individual microspheres and quantify the result of this based on fluorescent reporter signals.

CVD kit was used to measure TPAI-1, E-selectin, S-VCAM, S-ICAM for all samples using Luminex HCVD1-67AK kit. The procedure involves pre-wetting the filter paper of microtiter filter plate with 200 µl of 1X wash buffer, mixed well, sealed and placed on a plate shaker for 10 minutes at room temperature. Remove the wash buffer by vacuum and blot dry. Add the standards and control to respective wells. Add 25µl of assay buffer to all the sample wells. Add 25µl of the samples to the sample well. Vortex the beads and add 25µl of these beads and seal the plate and vortex on a plate shaker overnight (16-18hrs) at 4°C. Remove the excess fluid by vacuum and then
add 200µl of wash buffer twice with vacuum filtration. Add 25µl of detection antibodies and vortex the plate for 1 hour at room temperature. Now add 25µl of streptavidin-phycoerythrin mixture and then agitate for 30 minutes in room temperature. Gently remove the excess fluid by vacuum and then add 200µl of wash buffer twice with vacuum filtration after every wash. Lastly add 100µl of sheath fluid to each of the well and placed on plate shaker for 5 minutes and then placed on luminex 100™.

**DNA Extraction by phenol chloroform method**

**Principle**

Whole blood samples are used for DNA extraction by Phenol-chloroform method. To the blood sample double volumes of RBC lysis buffer was added and centrifuge at 2500rpm, wherein RBC, plasma can be separated and it can be repeated until all the RBC is lysed. Next digestion buffer and 10µl of proteinase K was added and incubate at 54°C for 2-3hrs until all the pellets break. Add 250µl of sodium perchlorate, phenol(500µl)and 500µl of Chloroform: isoamyl alcohol (25:24:1) ratio and centrifuge at 4000rpm for 15’ and the supernatant is again treated with chloroform and isoamyl alcohol(24:1)and centrifuged at 4000rpm. The aqueous phase is now mixed with approximately 5ml of chilled absolute alcohol. The DNA lump is pooled out and washed twice with 70% ethanol at 12000 rpm. DNA is dried and dissolved in 100µl of TE buffer and kept at 56°C for 1hr and stored at -80°C. (Sambrook et al 1989)

Details of buffer and extraction reagents used for DNA extraction
**RBC Lysis Buffer**

Sucrose    109.54 g

1M Magnesium Chloride 5ml

This is made up to 990ml with 1M Tris Hcl 10ml Milli Q water, autoclaved and 10ml of triton X added and made up to 1000ml.

**Digestion Buffer**

1M Tris Hcl

1M Sodium chloride

0.5 M EDTA (Na salt)

Made up to 95ml with Milli Q water, autoclaved, after reaching to room temperature 5ml of 20% S DS added.

**Tris EDTA buffer**

1M Tris Hcl (ph 8.0) 2ml

0.5 M EDTA 4ml

Made up to 200ml with Milli Q water

**Procedure**

- To the blood sample, 2 volumes of RBC lysis buffer was added and mixed gently by inverting the tube till it becomes transparent.

- It is centrifuged at 2500 rpm for 10 minutes to obtain a pellet. The supernatant containing the hemolysed RBC is discarded.

- The pellet was re suspended in RBC lysis buffer which equals to the initial blood volume and tapped gently to disturb the pellet.
• Again centrifuged at 2500 rpm for 10 minutes and the supernatant were discarded to obtain a clear white pellet. This procedure was repeated until we get a clear pellet which was free of RBC.

• The pellet was disturbed thoroughly and the digestion buffer which was half the volume of initial volume was added.

• Proteinase K (Sigma Aldrich, India) was added to the final concentration of 10µg/ml and Sodium Dodecyl Sulphate (SDS) was added to make 1% concentration in the final solution. It was mixed thoroughly by gently inverting the tube for 3-4 minutes till the solution became viscous and thereafter incubated in hot water bath at 56^0C for 4-5 hrs for proper digestion.

• When the solution became clear 5M sodium perchlorate (NaClO₄) which is one fourth the volume of digestion buffer was added and mixed gently for 3-4 minutes.

• Phenol (Tris saturated), chloroform and iso-amyl alcohol in 25:24:1 ratio was added in equal volume to a mixture of digestion buffer and 5M NaClO₄. After proper mixing for 1 minute it is centrifuged at 4000 rpm for 15 minutes at 4^0C.

• Aqueous layer was transferred carefully into another sterile polypropylene centrifuge tube using broad mouth tip. To this equal amount of chloroform and iso amyl alcohol in 24:1 ratio was added to the transferred supernatant and mixed gently for 3-4 minutes and centrifuged at 4000 rpm for 15 minutes at 4^0C. After centrifugation the aqueous phase was transferred to a freshly labeled tube.

• Exactly double the volume of chilled absolute alcohol was added and mixed gently by inverting the tube to precipitate the DNA.
DNA lump was spooled out into a fresh labeled 1.5ml tube to wash the DNA twice with 70% ethyl alcohol.

The pellets were dried at room temperature properly to ensure that whole alcohol was evaporated.

To this dried pellets add 100µl of TE. Keeping the tube at 56°C for 30-45 minutes will enhance the dissolution.

Store the DNA samples at 4°C or -20°C or -80°C as per the period of storage.

**Quantitative and qualitative check for extracted DNA**

**Quantification of DNA**

The extracted DNA was quantified by the spectrophotometer method followed by checking in 1% agarose gel (Maniatis et al., 1989)

**Tris acetate EDTA (TAE) buffer**

Tris 48.4g

Acetic acid 11.402ml

0.5M EDTA 20ml

Dissolved in 1 lt Miili Q water. 50ml of 20X buffer was made upto 1000ml with Milli Q water to obtain 1X TAE.

The entire extracted DNA was quantified using UV spectrophotometer. A ratio of OD values at 260nm and 280 nm indicates the purity of extracted DNA. If the ratio is in between 1.8-2.0 range, the DNA is considered free from protein and RNA contamination. A value above 2 shows RNA contamination and below 1.6 signifies
Protein impurities. Such samples need to be treated with RNase or Proteinase or Sodium acetate for better results.

Absorption spectrum of DNA is between 260 and 280nm. At 260nm absorbance of 1.00 OD, measured in a cuvette with 1 cm path length, is indicative that concentration of DNA is approximately 50µg/ml.

Concentration of DNA (µg/ml) = OD at 260nm x dilution factor x 50µg/ml.

**Loading dye (stock)**

Bromophenol blue 25mg

Xylene cyanol 25mg

Dissolved in 10 ml of milli q water. 5ml of 40% sucrose solution was added to 1ml of loading dye stock to make working solution.

**Ethidium Bromide solution (EtBr) (6%)**

0.6g ethidium bromide was dissolved in 2ml of milliq water and made up to 10ml.

All extracted DNA were run in agarose gel (2%.2.4gms of Agarose in 120ml of TAE Buffer) with 2µl of Ethidium bromide, wherein the 2µl of diluted DNA sample are added to bromophenol blue and loaded onto the gel to check the quality of DNA molecule and viewed under UV light to check the bands.

**Primer Designing and preparation**

Primers for the snp’s were designed using Primer 3 software. The desired snp region (around 1000bp) was selected from the ensemble and the designed primers
were checked for its quality in Net Primer and its similarity was noted in BLAST. The Forward and reverse oligonucleotides are sent to Applied Biosystem.

The customized primers are mixed with Milli Q as suggested in the technical data sheet. Keep it in dry bath at 55°C for 10’. Aliquot 4µl of prepared primer into 96µl of Milli Q.

**List of primers used in the present study**

<table>
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<tr>
<th>Gene</th>
<th>Primer rs ID</th>
<th>Base pair position</th>
<th>Position</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>APOA5</td>
<td>rs2075291</td>
<td>116661392</td>
<td>Exon 3 missense mutation</td>
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Genetic Study

PCR

Before starting PCR, gradient PCR are done to estimate the right annealing temperature for the primer. The Polymerase Chain Reaction (PCR) includes a master mix which is added along with DNA sample.

The master mix consists of

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<th>PCR Reagent</th>
<th>Quantity</th>
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<td>PCR Buffer</td>
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<td>DNTP’s</td>
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<tr>
<td>Forward Primer</td>
<td>0.18µl</td>
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<tr>
<td>Reverse Primer</td>
<td>0.18µl</td>
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<td>Taq Polymerase</td>
<td>0.1µl</td>
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<tr>
<td>Milli Q</td>
<td>7.04µl</td>
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<tr>
<td>Diluted DNA</td>
<td>1µl</td>
</tr>
</tbody>
</table>

The master mix is placed in the thermocycler at 94°C for 2’, 95°C for 1’, 58°C (depends on annealing temperature of primer) for 0.45’ and 72°C for 2.30’ 72°C for 7’ and 4°C for ∞ (Karl Mullis et al 1983) and placed in Gene amp 9600 thermo cycler. (Perkin-Elmer)
Cycle Sequencing

The PCR products are run in gel electrophoresis (2% agarose gel) and bright, thick bands are selected for sequencing. The sequencing reaction mixture consists of

- Big dye (0.5µl),
- Primer (0.2µl),
- Sequencing Buffer (1.75µl),
- Milli Q (7.05µL) and the PCR product (0.5µl).

They are run on a Gene amp 9600 thermo cycler (perkin- elmer) at 96°C for 0.10’, 55°C for 0.05’ and 60°C for 4.0’ and 4°C for ∞.

Sequencing reaction clean up

To these plates add 52µl each of washing buffer containing 5ml of 100% ethanol and 200µl of Sodium acetate Ph 5.2.

Seal the plates with an adhesive foil cover mix contents and incubate for 15 minutes at room temperature.

Centrifuge at 4000 rpm for 30 minutes at room temperature.

Remove the seal, decant the supernatant and centrifuge plate upside down at short spin (180rpm) for 30 seconds to remove all supernatants.

Add 100µl of 80% ethanol, seal the plate and centrifuge at 4000 rpm for 20 minutes at 15°C
Remove the seal, decant the ethanol, seal the plate and centrifuge plate upside down at 180 rpm for 30 seconds to remove all the ethanol.

Air-dry your plate for 10-15 minutes to ensure that all ethanol has been removed prior to sequencing set up.

**Sequencing set up for 3730 genetic analyser**

Add 10µl Hi-di formamide to each DNA pellet and seal the plate with septa.

Denature samples by heating to 96°C for 3 minutes in the thermocycler and immediately place on ice.

Prepare a sample sheet and create a plate record on the 3730 genetic analyser.

Place your plate into a cassette and load onto the ABI PRISM R 3730 and run the sequencing analysis. Once the analysis is complete, data can be analysed using seqscape program.

**DNA analyser ABI PRISM R 3730**

ABI PRISM R 3730 DNA automatically analyzes dna molecules labeled with multiple fluorescent dyes, it consists of a charge couple device (ccd) camera and a power computer that includes software for data collection and data analysis. After samples are loaded onto the system’s vertical gel, they undergo electrophoresis, laser detection and computer analysis. Eletrophoretic separation can be viewed on computer section.

**Sequence alignment**

The sequences which were generated align to the individual reference sequences with the use of seqscape v2.5 software. (Applied Biosystems,USA) Seqscape 2.5 is one of the software of applied Biosystems intended for automatic data analysis of sequences. It executes sequence assessment for variation identifications and
validation. It also permits re-sequenced data analysis, evaluating the consensus sequences to an identified reference sequences. The reference sequence for the sample can be acquired by NCBI.

To set an apparent range of data sequence, a process that believes quality values of the bases was used which removes bases from the sequence ends until less than 4 bases out of 20. Depending on the quality of sequence and specified criteria for filter the samples with low quality were not assembled by the software. The unassembled samples were re sequenced until it contented the quality.

**Statistical Analysis**

The data were arranged and checked twice before entering into the excel spreadsheet. All the variables result were expressed as mean, percentage and standard deviation. Data will be analyzed using SPSS version 12.0 software (SPSS, Chicago, IL, USA). The Student t-test is a statistical hypothetical test which is used to check the significant difference between the data, Chi square test is usually used for determining observed and expected frequency in one or more categories. ANOVA (Analysis of Variance) is a statistical model used to analyze the differences in group means in more than 3 groups. The pearson correlation test is used to check the linear dependence between variables. The pearson correlation co-efficient is expressed in r. The genetic data, allelic frequency of each of the snp’s and their odds ratio with the affected and unaffected subjects were determined using PLINK association analysis. Wherein PED file with sample ID, gender, disease status and genotype were given and the MAP file with chromosome number, rs ID and sample position were noted and loaded into PLINK file using MS DOS command function Plink –file name of file –assoc –ci 0.95 –assoc –noweb.