3.0 MATERIAL AND METHODS

3.1 Recruitment of Subjects

Three hundred patients, who were admitted in cardiology department for evaluation of chest pain and found angiography positive, were selected in the study consecutively.

3.2 Inclusion Criteria

All male and female found positive for angiography, age 25-90 years were included in the study.

3.3 Exclusion Criteria

Exclusion criteria were presence of chronic kidney disease, hepatic dysfunction, known endocrinal (except DM), rheumatological diseases, chronic infections or alcoholism. The inflammatory markers are significantly elevated in rheumatological disorders. Insulin secretion and synthesis are strongly influenced in endocrine disorders; Chronically ill patients have multiorgan dysfunction. Hence, these were excluded from the study.

3.4 Ethical Considerations

All individuals who participated in this project received verbal and written explanation in both English and Marathi languages. All subjects were asked to sign an informed consent form (provided in both English and Marathi) prior to the commencement of the interview. Anonymity of participants and confidentiality of all the data were guaranteed during the process of data collection. Permission to conduct the study was obtained from the Institutional ethical committee of Deenanath Mangeshkar Hospital, Pune.

3.5 Clinical data collection

All cases were interviewed using a questionnaire, which included data on smoking, physical activity. Data on clinical history of HTN and DM and medications (antihypertensive, lipid lowering and oral hypoglycemic agents) was also acquired.

3.6 Physical measurement

Physical measurement was done including height, weight, waist, and hip circumference. Waist-hip ratio (WHR) is the ratio of the circumference of the waist to that of the hips. It was calculated by measuring the waist circumference (midway between lowest part of lowest rib and upper part of iliac bone) and dividing by the hip circumference
at its widest part. BMI was calculated by dividing weight in Kg with square of height in meters.

3.7 Nutritional assessment

- Nutrition assessment was done once at the time of recruitment; based on previous two days 24 hour dietary recall. Mean of recall of two days was taken. Diet was assessed using a computer based comprehensive diet assessment known as Diet soft software, version: 1.1.7 [developed by Invincible IDEAS (www.invincibleideas.com) based on book Nutritive value of Indian Foods by C. Gopalan, B.V.Rama SastrI and S.C.Balasubramaniam, National Institute of Nutrition, Indian council of Medical Research, Hyderabad, India] (514).

- Questionnaires and diet assessments were administered via interview by trained staff. Portion size estimation was undertaken using volume measures, circular measures, numbers and linear measures. Mean of recall of two days was taken. To help participants estimate portion sizes, interviewers provided commonly used serving plates, bowls, utensils, cups and spoons. If measurements could not be given we recorded in three sizes: small, medium or large and any unusual intake was noted on the recall. For each nutrient, we used a standardized unit of measurement and reported values per 100 grams of edible portion of food product. There was no significant change in the dietary pattern of these participants and no change in lifestyle also.

3.8 Assessment of Prakriti among the subjects (Ayurvedic Principle)

- AyuSoft as used for assessment of prakriti in the subjects. It consists of questionnaire for the identification of prakriti. Questionnaires in Ayusoft were administered via interview by experienced staff under the guidance of vaidya. Accurate picture of prakriti was captured by the questionnaire. AyuSoft is a collaborative project between the Government of India’s Centre for Development of Advanced Computing (C-DAC) and the University of Pune. AyuSoft translates the logic of classical Ayurvedic texts into comprehensive, authentic, intelligent and interactive knowledge repositories with the help of complex analytical tools (http://ayusoft.cdac.in). The AyuSoft database includes more than 5 lakh records and information from nine texts; including the Brihadtrayee and Madhava Nidana.
standardized and validated software; which has been used by other authors in published studies (799-800) and being used in ongoing trial (805).

- The classification broadly takes into account features like body build, physiology, physical endurance, etc. Besides, the questionnaire also captures the information pertaining to history of disease. Each question has multiple options to choose from and each of the option further refers to it being a property attributed to Vata, Pitta or Kapha. Individuals who had thin and narrow body frame, weakly developed body build, with irregular appetite, food and bowel habits, difficulty in gaining weight, quick at physical activities, dry skin and hair, and less tolerance for cold temperature were considered as Vata Prakriti. Individuals with moderately developed build, high frequency of appetite and thirst, good digestive power, perspiration tendency higher than normal, tolerance for cold weather, moderately mobile with moderate physical strength were identified as Pitta Prakriti. Individuals who had broad body frames with well developed body build, tendency to gain weight, low appetite and digestion, preferred to be less mobile, less forgetful and with good healing power and cool temperament, were selected as Kapha individuals (791,792).

- Individual with dual constitution show a combination of two doshas (792). Scores for Kapha (K), Pitta kapha (PK), Vata Kapha (VK) and Vata Pitta (VP) were calculated with the help of software in consultation with ayurveda expert. Prakriti of these volunteers was designated as Vata-Pitta, Pitta-Kapha, or Vata-Kapha. While designating the Prakriti, the individuals with Vata as the primary Dosha and Pitta as the secondary Dosha (i.e., Vata-Pitta individuals) and the individuals with Pitta as the primary Dosha and Vata as the secondary Dosha (i.e., Pitta-Vata individuals) were considered to be equivalent and were grouped under Vata-Pitta. Similarly, the Pitta-Kapha individuals and the Kapha-Pitta individuals were treated to be equivalent and were grouped under Pitta-Kapha. On similar lines, the Vata- Kapha individuals and the Kapha-Vata individuals were treated to be equivalent and were grouped under Vata Kapha. This was done to avoid the necessity of creating six groups of dual Prakriti, which would have been a deviation from the Ayurveda textbooks.
3.9 Biochemical Investigations

- Fasting blood samples were collected after 14 hour fasting. Samples were kept at -60°C till analysis. Cholesterol, triglyceride, HDL were measured by using CHOD PAP, LIP/GK, enzymatic reaction was done by using RX Daytona (Randox Lab. India Pvt. Ltd) analyzer; LDL and VLDL were calculated by Freidwarld formula. Vitamin B12, Hcy, Folic acid and Insulin was done by Microparticle enzyme immunoassay (MEIA), Fluorescence polarization immunoassay (FPIA) and Ion Capture MEIA methods. TNF-α, hsCRP, IL6 were done by ELISA (Enzyme linked immunosorbent assay) method using commercial kits from Diaclone-France, Biocheck-USA, Randox-UK, Abbott-US by using instruments Axsym (Abbott Health Care,US), Daytona (Randox Laboratories,UK) and ELISA Reader (Merck,US)

- Insulin resistance and sensitivity was calculated by using HOMA model [HOMA-IR = fasting insulin (µIU/ml)*fasting glucose (mmol/l)/22.5] (806) and QUICKI [QUICKI= 1/(log(fasting insulin µU/mL)+log(fasting glucose mg/dL))] (807) respectively.

Biochemical analysis (808) was done as follows:

**Glucose**

Glucose-oxidase method (GOD- POD method): Glucose is oxidised in the presence of oxygen and GOD to give gluconic acid and hydrogen peroxide. The hydrogen peroxide formed reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to give red quinonemine. The intensity of colour is proportional to glucose concentration.

Enzymatic colorimetric determination of glucose according to the following reaction

\[
\text{Glucose Oxide} \\
\text{Glucose + O}_2 \xrightarrow{\text{Peroxidase}} \text{Gluconic acid+ H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{Phenol + 4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Red quinone + 4 H}_2\text{O}
\]

Linearity: The method is linear for up to a glucose value of 500 mg/dl.
Sensitivity: 10 mg/dl
Coefficient of variance: 2.85 %
Measuring interval: 10-500 mg/dl
Normal Range: 70-110 mg/dl (fasting), 120-140 mg/dl (post prandial)

**Urea**

U/V method: Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced in the reaction combines with alpha-oxoglutarate and NADH in the presence of glutamate-dehydrogenase to yield glutamate and NAD⁺

\[
\text{Urea} + \text{H}_2\text{O} \xrightarrow{\text{Urease}} 2\text{NH}_4^+ + \text{CO}_2
\]

\[
2\alpha-\text{oxoglutarate} + 2\text{NH}_4^+ + 2\text{NADH} \xrightarrow{\text{GLDH}} 2\text{L-glutamate} + 2\text{NAD}^+ + 2\text{H}_2\text{O}
\]

Linearity: 304 mg/dl.
Sensitivity: 9 mg/dl
Coefficient of variance: 3.44%
Measuring interval: 9-304 mg/dl
Normal Range: 10-45 mg/dl

**Creatinine**

Alkaline picrate method: The assay is based on the reaction of creatinine with sodium picrate. Creatinine reacts with alkaline picrate to form a red complex. The time interval chosen for measurements avoids interferences from other serum constituents. The intensity of the colour formed is proportional to the creatinine concentration in the sample.

Linearity: 22 mg/dl.
Sensitivity: 0.4 mg/dl
Coefficient of variance: 3.93%
Measuring interval: 0.4-22 mg/dl
Normal Range: 0.5-1.3 mg/dl (Male), 0.6-1.1 mg/dl (Female)

**Total Protein**

Biuret Method: In alkaline medium, peptide bonds of proteins react with cupric ions in biuret reagent to form violet coloured complex with an absorption maximum at 546 nm. The intensity of the colour formed is directly proportional to the concentration of total proteins in the sample.

Linearity: 13 gm/dl.
Sensitivity: 0.64 gm/dl  
Coefficient of variance: 3.28 %  
Measuring interval: 0.64-13 gm/dl  
Normal Range: 6.4-8.3 mg/dl

**Albumin**

BCG Method: Under acidic conditions, the albumin present in the sample binds to bromocresol green to form a green coloured albumin-BCG complex, which is photometrically measured at 578 nm. The intensity of the colour formed is directly proportional to albumin concentration in the sample.

Linearity: 5 gm/dl.  
Sensitivity: 0.5 gm/dl  
Coefficient of variance: 3.07 %  
Measuring interval: 0.5-5 gm/dl  
Normal Range: 3.2-4.8 mg/dl

**Total Bilirubin**

Sulphanilic acid-DMSO (End Point method): Bilirubin in the presence of sulphanilic acid diazonium salt forms a red coloured azo compound in alkaline solutions. The total bilirubin in serum is determined using the method of Jendrassik and Gorf by coupling it with diazoitated sulphanilic acid after the addition of DMSO. The intensity of the colour is proportional to bilirubin concentration.

Linearity: 20mg/dl.  
Sensitivity: 0.3mg/dl  
Coefficient of variance: 3.63 %  
Measuring interval: 0.3-20 mg/dl  
Normal Range: 0.3-1.2 mg/dl

**Direct Bilirubin**

DMSO-Colorimetric method: Bilirubin is converted to coloured azobilirubin by diazotized sulfanilic acid and measured photometrically. Of the two fractions present in serum, bilirubin-glucuromide and free bilirubin loosely bound to albumin, only the former reacts directly in aqueous solution (bilirubin direct), while free bilirubin requires solubilization.
with dimethylsulphoxide to react (bilirubin indirect). The intensity of the colour formed is proportional to the direct bilirubin concentration in the sample.

Linearity: 15mg/dl.
Sensitivity: 0.3mg/dl
Coefficient of variance: 8.43%
Measuring interval: 0.3-15mg/dl
Normal Range: <0.3 mg/dl

**ALT (Alanine aminotransferase)**

Kinetic method: 2-oxoglutarate reacts with L-alanine in the presence of the enzyme ALAT to give glutamate and pyruvate. Pyruvate formed undergoes dehydrogenation in the presence of NADH and enzyme LDH to give lactate and NAD. The rate of NADH consumption is measured photometrically and is directly proportional to ALAT activity in the sample.

\[
\begin{align*}
A\text{-}oxoglutarate + L\text{-}alanine & \xrightarrow{ALT} L\text{-}glutamate + pyruvate \\
Pyruvate + NADH + H^+ & \xrightarrow{LDH} L\text{-}lactate + NAD^+
\end{align*}
\]

Linearity: 600 U/L.
Sensitivity: 10 U/L
Coefficient of variance: 4.49%
Measuring interval: 10-600 IU/L
Normal Range: 10-40 IU/L

**AST (Aspartate Aminotransferase)**

Kinetic method: 2-oxoglutarate reacts with L-aspartate in the presence of the enzyme ASAT to give glutamate and oxaloacetate. Oxaloacetate formed undergoes dehydrogenation in the presence of NADH and enzyme MDH to give malate and NAD. The rate of NADH consumption is measured photometrically and is directly proportional to ASAT activity in the sample.

\[
\begin{align*}
A\text{-}oxoglutarate + L\text{-}aspartate & \xrightarrow{AST} L\text{-}glutamate + Oxaloacetate \\
Oxaloacetate + NADH + H^+ & \xrightarrow{MDH} L\text{-}malate + NAD^+
\end{align*}
\]
Linearity is 600 U/L
Sensitivity: 10 U/L
Coefficient of variance: 4.21%
Measuring interval: 10-600 IU/L
Normal Range: 20-40 IU/L

**Alkaline phosphatase**

PNP-DEA buffer method: 4-nitrophenylphosphate undergoes hydrolysis in the presence of enzyme alkaline phosphatase to give phosphate and 4-nitrophenol. The rate of increase in 4-nitrophenol is determined photometrically and is directly proportional to the ALP activity in the sample.

\[
p\text{-nitrophenyl phosphate} + H_2O \rightarrow \text{phosphate} + p\text{-nitrophenol}
\]

Linearity: 2000U/L.
Sensitivity: 36U/L
Coefficient of variance: 3.92%
Measuring interval: 36-2000U/L
Normal Range: 100-390 IU/L

**Cholesterol**

CHOD-PAP Method: Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyses the esters. In the subsequent enzymatic oxidation by cholesterol oxidase, hydrogen peroxide is formed. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

\[
\begin{align*}
\text{Cholesterol esterase} & : \\
\text{Cholesterol ester} + H_2O & \rightarrow \text{Cholesterol} + \text{Fatty acids} \\
\text{Cholesterol oxidase} & : \\
\text{Cholesterol} + O_2 & \rightarrow \text{Cholestene-3-one} + H_2O_2 \\
\text{peroxidase} & : \\
2H_2O_2 + \text{phenol} + 4\text{-Aminoantipyrine} & \rightarrow \text{quinoneimine} + H_2O
\end{align*}
\]
Linearity: 643 mg/dl
Sensitivity: 10 mg/dl
Coefficient of variance: 3.36%
Measuring interval: 10-643 mg/dl
Normal Range: below 200 mg%-desirable, 200-240 mg%-boderline, above 240 mg%-undesirable

**HDL Cholesterol**

Enzymatic Clearance Assay method: The assay consists of two distinct reaction steps: elimination of chylomicron, VLDL cholesterol and LDL cholesterol by cholesterol esterase, cholesterol oxidase and subsequently catalase. The second step involves the specific measurement of HDL cholesterol after the release of HDL cholesterol by detergents in Reagent 2.

1. Elimination of chylomicron, VLDL-Cholesterol and LDL-Cholesterol by cholesterol esterase, cholesterol oxidase and subsequently catalase

\[
\begin{align*}
\text{Cholesterol ester} & \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol + fatty} \\
\text{Cholesterol + O}_2 & \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestenone + H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 & \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O}_2 + \text{O}_2
\end{align*}
\]

2. Specific measurement of HDL-Cholesterol after release of HDL-Cholesterol by detergents in Reagent 2.

\[
\begin{align*}
\text{Cholesterol ester} & \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol + fatty} \\
\text{Cholesterol + O}_2 & \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestenone + H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-AA+HDAOS} & \xrightarrow{\text{Peroxidase}} \text{Quinone pigment + 2 H}_2\text{O}
\end{align*}
\]

Linearity: 144 mg/dl.
Sensitivity: 1.43 mg/dl
Coefficient of variance: 5.03%
Measuring interval: 1.43-144 mg/dl
Normal Range: >60 mg%-desirable, 40-59 mg% borderline, <40 mg%-undesirable
**Triglyceride**

GPO-PAP end point method: TGs in the presence of lipase enzyme forms glycerol, fatty acid. Glycerol undergoes phosphorylation in the presence of ATP to form glycerol -3-phosphate which undergoes oxidation to form DHAP, which reacts with chloroprene and 4-aminophenazone in the presence of POD to form quinoneimine. The colour is proportional to TG concentration.

\[\text{TG} + \text{H}_2\text{O} \xrightarrow{\text{Lipases}} \text{glycerol} + \text{fatty acids}\]

\[\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate + ADP}\]

\[\text{Glycerol-3-phosphate + O}_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone+ phosphate + H}_2\text{O}_2\]

\[2\text{H}_2\text{O}_2 + 4\text{-aminophenazone} + 4\text{-chlorophenol} \xrightarrow{\text{POD}} \text{quinoneimine + HCL + 4H}_2\text{O}\]

Linearity: 1133 mg/dl.

Sensitivity: 12 mg/dl

Coefficient of variance: 4.25%

Measuring interval: 12-1133 mg/dl

Normal Range: below150 mg%-desirable, 150-499mg%-borderline, above500 mg%-undesirable

**Uric Acid**

Phosphomolydate UV Method: Uric acid is converted by uricase to allantoin and hydrogen peroxide, which under the catalytic influence of peroxidase, oxidizes TOOS and 4-aminophenazone to form a red-violet quinoneimine compound.

\[\text{Uric acid} + \text{O}_2 + 2\text{H}_2\text{O} \xrightarrow{\text{Uricase}} \text{Allantoin} + \text{CO}_2 + \text{H}_2\text{O}_2\]

\[2\text{H}_2\text{O}_2 + \text{H}^+ + \text{TOOS} + 4\text{-Aminophenazone} \xrightarrow{\text{peroxidase}} \text{quinine di-imine dye} + 4\text{H}_2\text{O}\]

TOOS: N-ethyl-N-(2-Hydroxy-3-sulfopropyl)-3-methylaniline

Linearity: 23.1mg/dl.

Sensitivity: 0.3 mg/dl

Coefficient of variance: 3.02%

Measuring interval: 0.3 – 23.1 mg/dl

Normal Range: 3.5-7.2 mg/dl (Male), 2.6-6.0(Female)
**Calcium**

Arsenazo III colorimetric method: At a neutral pH, calcium forms a coloured complex with arsenazo III and the colour intensity of this complex is directly proportional to the concentration of calcium in the sample.

\[ \text{Ca}^{++} \text{ Arsenazo III} \rightarrow \text{Coloured complex} \]

Linearity: 17.6 mg/dl.
Sensitivity: 0.5 mg/dl
Coefficient of variance: 2.18 %
Measuring interval: 0.5-20 mg/dl
Normal Range: 9-11 mg/dl

**Phosphorus**

Phosphomolybdate UV Method: Ammonium molybdate reacts in acid medium in the presence of phosphorous to give phosphor-inorganic molybdate complex with yellow colour, which is measured at 340nm and the intensity of the colour formed is proportional to the inorganic phosphorous concentration in the sample.

Linearity: 24.6 mg/dl.
Sensitivity: 0.5 mg/dl
Coefficient of variance: 2.39 %
Measuring interval: 0.5 – 24.6 mg/dl
Normal Range: 2.7-4.5 mg/dl (Male), 2.8-4.0 mg/dl (Female)

**Magnesium**

Xylidil blue method: Magnesium ions react with xylidyl blue in an alkaline medium to form a water soluble purple-red chelate, the colour intensity of which is proportional to the concentration of magnesium in the sample. Calcium is excluded from the reaction by complexing with EGTA.

Linearity: 8 mg/dl.
Sensitivity: 0.68 mg/dl
Coefficient of variance: 4.27 %
Measuring interval: 0.68-8.0 mg/dl
Normal Range: 1.6-2.6 mg/dl
**Vitamin B12**

MEIA (Microparticle Enzyme Immunoassay) method: Sample and Vit B12 reagent are pipetted into the RV. RV is transferred to processing center. Sample anti Vit B12 alkaline phosphatase conjugate and anti Vit B12 coated microparticles are pipetted into one well of RV. The Vit B12 binds to both enzyme labeled antibody and the antibody coated microparticles. An aliquot of antibody-antigen-antibody complex is transferred to matrix cell. The complex is washed with buffer. The substrate 4-Methylumbelliferyl phosphate is added and the fluorescent product is measured by MEIA optical assembly.

Performance specification:
Linearity: 1200 pg/ml.
Sensitivity: 60 pg/ml
Coefficient of variance: 6.46%
Measuring interval: 60-1200 pg/ml
Normal Range: 208-960 pg/ml

**Homocysteine**

FPIA (Fluorescence Polarization Immunoassay) method: Sample, pre-treatment solution, Hcy antiserum and buffer are pipetted into one well of R.V. An aliquot of predilution mixture is transferred to the cuvette of R.V. The pretreatment solution removes the Hcy from binding sites on TBG, albumin and prealbumin. The RV is transferred to the Processing Centre. A second aliquot of the predilution mixture is transferred to the cuvette along the Hcy fluorescein tracer. Hcy and labeled tracer compete for the sites on the antibody molecules. The intensity of polarized light is measured by FPIA optical assembly.
Linearity: 50 µmol/L.
Sensitivity: 2.5 µmol/L
Coefficient of variance: 6%
Measuring interval: 2.5-50 µmol/L
Normal Range: 6-20 µmol/L

**Folate**

Ion capture MEIA (Microparticle Enzyme Immunoassay) method: Sample and folate reagent are pipetted into the RV. RV is transferred to processing center. Sample anti folate alkaline phosphatase conjugate and anti folate coated microparticles are pipetted into one
well of RV. The Vit folate binds to both enzyme labeled antibody and the antibody coated microparticles. An aliquot of antibody-antigen-antibody complex is transferred to matrix cell. The complex is washed with buffer. The substrate 4-Methylumbelliferyl phosphate is added and the fluorescent product is measured by MEIA optical assembly.

Linearity: 20 ng/ml
Sensitivity: 0.9 ng/ml
Coefficient of variance: 8%
Measuring interval: 0.9 – 20 ng/ml
Normal Range: 3-17 ng/ml

**HbA1c**

Boronic acid affinity assay: The reagent contains agents that lyse erythrocytes and precipitate haemoglobin specifically, as well as blue boronic acid conjugate haemoglobin. When blood is added to the reagent, the erythrocytes immediately lyse. All haemoglobin precipitates. The boronic acid conjugate binds to the cis-diol configuration of glycated haemoglobin. An aliquot of the reaction mixture is added to the test device and all the precipitated haemoglobin, conjugate-bound and unbound, remains on the top of the filter. Any excess of coloured conjugate is removed with the washing solution. The precipitate is evaluated by measuring the blue (glycated hemoglobin) and the red (total hemoglobin) colour intensity, the ration between glycosylated Hb and total Hb, then being proportional to the percentage of HbA1c in the sample.

Linearity: 18%
Sensitivity: 2.5%
Coefficient of variance: 5%
Measuring interval: 2.5-18%
Normal Range: 2.5-6.3%

**Insulin**

The AxSYM Insulin assay is based on the Microparticle Enzyme Immunoassay (MEIA) technology. Sample and all AxSYM Insulin assay reagents required for test are pipetted into wells of the reaction vessel. The RV immediately transferred into the Processing Center. Sample, Anti-Insulin (Mouse, Monoclonal) Coated Microparticles and Assay Buffer are pipetted to one well of the reaction vessel. During the incubation of this reaction
mixture the Insulin in the specimen binds to the Anti-Insulin Coated Microparticles forming an antibody-antigen complex. An aliquot of the reaction mixture is transferred to the matrix cell. The microparticles bind irreversibly to the glass fiber matrix. The matrix cell is washed to remove unbound materials. The Anti-Insulin (Mouse, Monoclonal) Alkaline Phosphatase Conjugate is dispensed onto the matrix cell and binds to the antibody-antigen complex. The matrix cell is washed to remove unbound materials. The substrate, 4-Methylumbelliferyl Phosphate, is added to the matrix cell and the rate of fluorescent product formation is measured by the MEIA optical assembly.
Linearity: Linearity is 300 μU/ml
Sensitivity: 1.0 μU/ml
Coefficient of variance: 2.9 %
Measuring interval: 1 to 300 ng/ml
Normal Range: 8-15 μu/L

**Interleukin-6**

A capture Antibody highly specific for IL-6 has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of IL-6 samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti-IL-6 secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of IL-6 present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of IL-6 in any sample tested.
Linearity: 200 pg/ml
Sensitivity: 2 pg/ml
Coefficient of variance: 4.2% (intra assay), 7.7% (inter assay)
Measuring interval: 2-200 pg/ml
Normal Range: <2 pg/ml

**Tumor Necrosis Factor-α**

A capture Antibody highly specific for TNF-alpha has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of TNF-alpha samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti-TNFα secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of TNF-alpha present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of TNF-alpha in any sample tested.

Linearity: 800 pg/ml
Sensitivity: 8 pg/ml
Coefficient of variance: 3.3% (intra assay), 9.0 (intra assay)

Measuring interval: 8-800 pg/ml
Normal Range: <8 pg/ml

**Highly Sensitive C-Reactive Protein**

The hsCRP ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the on the CRP molecule. This mouse monoclonal anti-CRP antibody is used for solid phase immobilization (on the microtiter wells). A goat anti-CRP antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the two antibodies, resulting in the CRP molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 45- minute incubation at room temperature, the wells are
washed with water to remove unbound labeled antibodies. A tetramethylbenzidine (TMB) reagent is added and incubated for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl changing the color to yellow. The concentration of CRP is directly proportional the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

Linearity: 119.3 mg/l
Sensitivity: 0.1 mg/l
Coefficient of variance: 7.5% (intra assay), 4.1% (inter assay)
Measuring interval: 0.1-119.3 mg/L
Normal Range: 0.068-8.2 mg/l

3.10 Definitions

- Family history: Family history was considered positive if any first degree male had evidence of CVD before the age of 55 years or female before the age of 65 years according to current guideline (132).
- Dietary habit: Non-vegetarians and semi-vegetarians were defined as those consuming animal products such as meat, poultry, fish and other sea foods more and less than once a week respectively. Patients who never consumed animal products like meat, poultry, fish and other sea foods, but were taking milk products were defined as vegetarians (809).
- WHR >0.9 for men and >0.85 for women used as measures of central obesity (810): BMI <25-Normal, ≥25 Overweight/ Obese (811).
- Atherogenic dyslipidemia was defined as TG level>150mg/dl and HDL Cholesterol level<40 mg/dl (812). Conventional risk factors were defined as follows: DM (by history, treatment or ADA criteria if newly detected (813), HTN (systolic and diastolic BP above 140 and 90 mmHg, respectively).
- Metabolic syndrome was defined according to International Diabetes Federation (IDF) criteria as follows: central obesity (waist circumference: male > 90 cm, female > 80 cm) plus any two: raised triglycerides (>150 mg/dl), reduced HDL cholesterol (<40 mg/dl in men or <50 mg/dl in women), raised blood pressure (systolic ≥ 130 mmHg or diastolic ≥ 85 mmHg), or raised fasting plasma glucose (fasting plasma glucose ≥ 100 mg/dl).
For comparison we have divided all subjects into three groups according to vitamin B12 levels; <100 pg/ml (group 1), 100-200 pg/ml (group 2), >200 pg/ml (group 3) and two groups according to Hcy levels; ≤ 15 µmol/l (group 1), >15 µmol/l (group 2) indicating hyperhomocysteinemia.

Patients were divided into three groups according to serum magnesium levels; group 1: ≤ 1.6 mg/dl (n=176, 58.6%), group 2: >1.6-2.6 mg/dl (n=102, 34%) and group 3: >2.6 mg/dl (n=26, 8.6%) and into two groups according to Recommended Dietary Allowance of magnesium; group 1 with dietary intake of magnesium ≤ 350 mg/day (n = 186, 62%) and group 2 (n = 114, 38%) with dietary intake of magnesium levels > 350 mg/day.

Physical activity: For the purpose of the statistical analyses and to conform to the National Heart Foundation protocol (814), participants were considered to have engaged in exercise if they walked at least 4 hours per fortnight, or engaged in several sessions of 20 minute duration of vigorous exercise with a total of 2 hours or more per fortnight, or engaged for 2 hours or more in housework activities or gardening that made them breathe hard or puff per fortnight, or if they engaged in 3 hours or more of less vigorous exercise per fortnight.

3.11 Duration of the study

The duration of this study was of 24 months, from year 2009 to 2011.

3.12 Statistical method

Statistical analysis was carried out using EPI Info, version 3.5.3 (CDC; Atlanta; USA) and SPSS 20. Data were presented as mean ± SD, median (range) or number (%) unless specified. All parametric data were analysed by student’s t-test if grouped in two group and ANOVA if > 2 groups. If Barlett’s chi-square test for equality of population variances was < 0.05 then Kruskal-Wallis test was applied. All non parametric data were analysed by chi-square test. Pearson correlation coefficient was used to assess relation between two quantitative risk factors. Multivariate linear regression analysis was utilized to examine the association between two quantitative parameters. Outcome variable is considered as dependant variable and adjustments were done for age, sex, BMI and various risk factors as required to measure association with independent variable. A p value of < 0.05 was considered statistically significant.