Materials and Methods
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Study type: The study was designed as a community based cross sectional, case-control study.

Duration of the study: 2½ years for data collection and 1½ years for compilation of the data.

Study setting: Kasturba Medical College, Dept. of Biochemistry and Clinical Biochemistry section at Kasturba Medical College Hospital, Ambedkar Circle (KMCHAC), Mangalore.

Selection of subjects:

Subjects were chosen from the local population which consists of a harmonious blend of Hindus, Muslims and Christians. No selection bias was introduced with regards to the religion of the individual. The religion and caste were however noted to explore the possibility as to a greater susceptibility of a particular community to the development of diabetes.

The selection was based on the ‘family history of diabetes in the first degree relatives’ (FDRs). ‘First degree relatives’ was defined as either parents or siblings of the participating subjects.

All the participating subjects had to be non-diabetic and those with a family history (FH+) were age matched with those without a family history (FH>). FH was ascertained only on the history given by the participants and no formal testing was done in the parents or siblings. Subjects reporting a negative family history were carefully questioned. Only those who definitely knew that their parents or siblings were undergoing regular medical checkup and were not diagnosed with diabetes were included. In case, the parents or any siblings had deceased, the cause of death was noted and it was ascertained that their glucose levels were in the normal range, particularly if it was an institutional death related to sickness.
Since the selection of the subjects depended solely on their family history of diabetes, an unintentional bias crept in and a section of the population who were educated, had some knowledge about diabetes and definitely knew their family history were selected. The subjects were chosen to fall in the age group of 20-60 years.

Subjects were identified only by word of mouth. Initially, the college and hospital teaching staff, their family members, the lab technicians and their family members etc. were invited to participate. The suitability of their inclusion was decided after detailed collection of personal and FH particulars. Later, their friends, relatives, colleagues etc. joined the study. A copy of the lab report pertaining to total leucocyte count, differential leucocyte count, ESR, fasting plasma glucose, 2hr 75gm postload glucose and fasting lipid profile was issued to all the participants with counseling wherever required and appropriate.

Ethical clearance:

The Institutional Ethics Committee of Kasturba Medical College, Mangalore reviewed and approved the protocol, the questionnaire (Annexure-1) and the consent form (Annexure-2) to be used for the study vide letter dated 11.11.2005 (Annexure-3).

Funding:

Funds for the study were received from the Department of Science & Technology, Ministry of Science & Technology, New Delhi under the ‘SERC Fast Track Scheme for Young Scientists’, vide sanction letters SR/FT/L-127/2005 dated 14.07.2006 and SR/FTP/L-127/2005 dated 29.10.2007.

Study design:

All the study participants were expected to be nondiabetic subjects. To ensure this, the subjects were asked lead questions as to whether they had previously got their blood glucose checked, if so, when was the test done,
what was the reason for getting the test done and the test values (details in questionnaire). Those with the family history (FH+) of diabetes served as the test group and were compared with those without a family history (FH-).

The two groups FH+ and FH- were to be further dichotomized based on the age. Hence, the younger subjects aged 20-39 years with a positive family history were termed FHY+ and the older subjects aged 40-60 years with a positive family history were termed FHE+. Similarly, the age matched subjects with a negative FH were termed FHY- and FHE- respectively. The sample size was calculated as under and it was decided to include 75 subjects in each of the groups; FHY+, FHE+, FHY- and FHE- to still have a good number expecting upto 20% exclusion or nonconforming subjects.

Sample size calculation:

Assuming 95% confidence interval, 80% power of the study and 10% non-responders/ exclusions, the sample size calculated was :

\[ n = \frac{Z^2_{\alpha} \cdot p \cdot q}{L^2} \]

\[ = (1.96)^2 \times 60 \times 40 \]

\[ = \left( \frac{60 \times 20}{100} \right)^2 \]

\[ = 9219.84 \]

\[ = \frac{9219.84}{144} \]

\[ = 64 + 10\% \text{ non-responders/exclusions} \]

\[ = 64 + 6 \]

\[ = 70 \]

Each of the four groups; FHY+, FHE+, FHY- and FHE- were to consist of 70 subjects.

Inclusion criteria:

Apparently healthy, as yet undiagnosed FDRs of type 2 diabetes patients of either sex in the age group 20-60 years.
Exclusion criteria:

Diabetes patients, pregnant women, chronic alcoholics, patients with known chronic inflammatory diseases and subjects with history of active infection in the previous three months were not enrolled in the study.

Wherever, a suitable subject had an inter-current acute illness, a later date was given for blood sampling so as to fulfill the inclusion criteria. Even when a candidate was not found suitable, the basic lab tests stated above were done and the copies of reports were issued to them. The special tests meant to meet the objectives of this study were not performed in their blood samples and such subjects were not included in the study. Other than paying commutation charges to some of the subjects and providing breakfast to most of the participants after the study time, no other financial coercions were used during the study.

All subjects voluntarily and of their free will participated in the study after having been explained the purpose of the study and upon signing the consent form. With the help of a questionnaire, the subjects’ family history, personal history, diet history and medical history were collected.

The participants were asked to come to KMCHAC, clinical Biochemistry lab, in the morning of a mutually convenient stipulated day, in light clothes after a well rested night’s sleep and an overnight fast of not less than 10 hrs and not more than 12 hrs.

Demographic details were collected in all the subjects meeting the inclusion criteria. A total of 301 subjects participated in the study.
Demographic details

- Age
- Height (Ht)
- Weight (Wt)
- Waist Circumference (WC)
- Hip Circumference (HC)
- Blood Pressure (BP)
- Waist-Hip Ratio (WHR)
- Body Mass Index (BMI)

Biochemical investigations

- Fasting Plasma Glucose (FPG)
- 2 hr postload glucose (2 hr PG)
- Glycated Haemoglobin (HbA1c)
- Fasting Insulin (FI)
- Homeostasis Model Assessment – Insulin Resistance (HOMA-IR)
- Fasting Lipid Profile (FLP)
- Total Leucocyte count (TLC)
- Erythrocyte Sedimentation Rate (ESR)
- Highly Sensitive C-reactive Protein (hsCRP)
- Ceruloplasmin
- Haptoglobin
- Fibrinogen
In 47 FH⁺ and 48 FH⁻ subjects selected randomly from the above groups, the following additional tests were performed.

- Morning Serum Cortisol
- Interleukin-6 (IL-6)

Methodology:

1. Age: Age was recorded as completed years.

2. Height (Ht): Ht was measured in centimeters (cm) using a stadiometer to the nearest cm and converted into meters (m).

3. Weight (Wt): Wt was measured in kilograms (kg) using a standard bathroom weighing scale recorded to the nearest 0.5 kg.

4. Waist circumference (WC): WC was measured in cm using a standard nonstretchable tailor’s tape midway between the last rib and the iliac crest, on bare skin to the nearest 0.5 cm.

5. Hip circumference (HC): HC was measured in cm using standard nonstretchable tailor’s tape at the widest part of the hips with light clothing on, to the nearest 0.5 cms.

6. Blood pressure (BP): Systolic BP (SBP) and diastolic BP (DBP) were separately noted to the nearest 2 mmHg, using a standard clinical mercury sphygmomanometer. BP was recorded after the subjects were rested for a minimum of 15 minutes and average of two readings at 5 minutes’ interval was considered.

7. Waist hip ratio (WHR): WHR was calculated as, \[
\frac{WC \text{ in cm}}{HC \text{ in cm}}
\]

8. Body mass index (BMI): BMI was expressed as kg/m² and calculated as, \[
\frac{Wt \text{ in kg}}{Ht \text{ in m}^2}
\]
Kits for all biochemical estimations were purchased from ‘Roche Diagnostics’ (Mannheim, Germany). Chemical analysis were performed on Hitachi 917 (Mannheim, Germany) auto analyser and immunoassay estimations were carried out on Elecsys 2010 (Mannheim, Germany). Respective calibrators were used for calibration of test with each batch of samples analysed. Manufacturer’s instructions described in the kit inserts of the corresponding kits regarding assay procedure, sample selection and storage conditions were followed.

Fasting plasma glucose (FPG): FPG was estimated by the glucose oxidase-peroxidase method. The enzyme glucose oxidase oxidizes glucose to gluconic acid and Hydrogen peroxide. The nascent oxygen released from H$_2$O$_2$ is further made to react with 4-aminoantipyrine and phenol by peroxidase to give a red quinone (quinoneimine).

Measuring range: 1-500 mg/dL.
Inter and intra assay CV: 0.69-1.74%.

9. 2 hr post load plasma glucose (2 hr PG): All subjects were given 75 gm D-glucose (Dabur, India) dissolved in 200 ml water and were asked to consume it within 5 minutes. The time was noted and exactly 2 hrs later, blood was drawn for 2 hr PG. For 2 hr PG estimation, the principle was similar to that of FPG.

10. HbA$_{1c}$: Glycated Hb (HbA$_{1c}$) was determined based on the turbidimetric inhibition immune assay (TINIA) for hemolysed whole blood. Tetradecyl trimethyl ammonium bromide was used as a detergent in the hemolysing reagent to eliminate interference from leucocytes. HbA$_{1c}$ in the sample reacts with anti HbA$_{1c}$ antibody to form soluble antigen-antibody complexes. On addition of polyhaptens, they react with excess anti-HbA$_{1c}$ antibodies to form an insoluble antibody-polyhapten complex which can be determined turbidimetrically. The Hb concentration is determined in a second channel. Liberated Hb in the hemolysed sample is converted to a derivative having a characteristic absorption spectrum which was measured bichromatically.
11. Fasting insulin (FI
): Electrochemiluminescence immunoassay. Biotinylated monoclonal insulin specific antibody and a monoclonal insulin specific antibody labeled with a ruthenium complex was incubated with 20 µL sample containing insulin to form a sandwich complex. After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured on to the surface of the electrode. Unbound substances are removed with procell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is instrument specific generated by 2-point calibration and a master curve provided via the reagent barcode.
Measuring range: 0.2-1000 µu/mL.
Inter and intra assay CV: 0.7-4.9%.

12. Homeostasis model assessment-Insulin resistance (Homa-IR): Insulin resistance was calculated as HOMA-IR using the formula\textsuperscript{119},
\[
\text{Homa-IR} = \frac{\text{Fasting Insulin (µu/mL)} \times \text{Fasting glucose (mg/dL)}}{405}
\]

13. Fasting lipid profile (FLP): This consists of Total Cholesterol (TC), Triacylglycerols (Tg), High density lipoprotein cholesterol (HDL-C), Low density lipoprotein cholesterol (LDL-C), Very low density lipoprotein cholesterol (VLDL-C) and TC/HDL-C ratio.

TC: Estimated enzymatically using cholesterol esterase and cholesterol oxidase. Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Free cholesterol is converted by oxygen, with the aid of cholesterol oxidase to Cholest-4-en-
3-one and H$_2$O$_2$. The H$_2$O$_2$ forms a red dye stuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. Measuring range: 3-800 mg/dL. Inter and intra assay CV: 0.7-2.7%.

Tg: Estimated by colorimetic enzymatic method. Tgs are hydrolysed by lipoprotein lipase to glycerol and fatty acids. Glycerol is phosphorylated by glycerol kinase and ATP to glycerol-3-phosphate which is oxidized to dihydroxyacetone phosphate and H$_2$O$_2$ by glycerol peroxidase. The H$_2$O$_2$ combines with 4-aminophenazone and 4-chlorophenol under the action of peroxidase to give 4-(p-benzoquinone-monoimino)-phenazone, H$_2$O and HCl. Measuring range: 4-1000 mg/dL. Inter and intra assay CV: 0.9-2.4%.

HDL-C: The cholesterol concentration of HDL-C was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with polyethylene glycol to the amino groups. Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of O$_2$, cholesterol is oxidized by cholesterol oxidase to $\Delta^4$-cholestenone and H$_2$O$_2$. In the presence of peroxidase, the H$_2$O$_2$ reacts with 4-aminoantipyrine and Sodium N-(2-hydroxy-3-sulphopropyl)-3, 5-dimethoxyanalanline to form a purple dye. Measuring range: 3-120 mg/dL. Inter and intra assay CV: 0.6-1.8%.

LDL-C: It was calculated by the Friedwald’s formula as,
\[
LDL-C = TC - \left( HDL-C + \frac{Tg}{5} \right)
\]

VLDL-C: It was calculated as, \[
\frac{Tg}{5}
\]

TC/HDL-C: It was calculated as, \[
\frac{TC}{HDL-C}
\]
14. Total leucocyte count (TLC): TLC was counted on Sysmex XT-1800 (Kobe, Japan) automated cell counter by flow cytometry and results expressed as cells/mm$^3$.

15. Erythrocyte sedimentation rate (ESR): ESR was measured by modified Westergren’s method automated on VES-matic 20 and cells in mm after 1$^{st}$ hour was noted.

16. hsCRP: Particle enhanced immunoturbidimetric assay. Anti CRP antibodies coupled to latex microparticles react with the antigen in the serum to form an antigen/antibody complex. Following agglutination, this was measured turbidimetrically.
   Measuring range: 0.1-20 mg/L.
   Inter and intra assay CV: 0.28-5.7%.

17. Ceruloplasmin: Immunoturbidimetric assay principle is employed. Anticerulplasmin antibodies react with antigen in the sample to form antigen/antibody complexes which, following agglutination, was determined turbidimetrically.
   Measuring range: 10-140 mg/dL.
   Inter and intra assay CV: 0.9-2.5%.

18. Haptoglobin: Immunoturbidimetric assay. Antiaptoglobin antibodies react with haptoglobin in the sample to form antigen/antibody complexes which after agglutination was determined turbidimetrically.
   Measuring range: 20-400 mg/dL
   Inter and intra assay CV: 1.4 6.9%.

19. Fibrinogen: The Clauss clotting time method was used for the determination of fibrinogen levels. Excess bovine thrombin was used to clot diluted plasma (1/10). The clot is detected photo-optically. The clotting time obtained is inversely proportional to the fibrinogen content. The results are interpolated from a standard curve prepared using a
reference plasma of known fibrinogen content at dilutions of 1/5, 1/10, 1/20 and 1/40.
Measuring range: 10-800 mg/dL.

20. Serum cortisol: Sample for the estimation of serum cortisol was collected between 6.30 and 8.30 a.m. and the principle employed was that of competitive immunoassay. Sample is first incubated with a cortisol specific biotinylated antibody and a ruthenium complex labeled cortisol derivative, when cortisol in the sample forms the immune complex. The 2nd incubation is with streptavidin-coated microparticles when the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with procell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
Measuring range: 0.018-63 µg/dL.
Inter and intra assay CV: 1.0-1.6%.

22. Interleukin-6 (IL-6): Estimated by electro chemiluminescent assay. Sample is incubated with a biotinylated monoclonal IL-6 specific antibody. After addition of a monoclonal IL-6 specific antibody labeled with a ruthenium complex and streptavidin coated microparticles the antibodies form a sandwich complex with the antigen of the sample. The microparticles are magnetically captured onto the surface of the electrode and application of a voltage to the electrode then induces chemiluminescence which is measured by a photomultiplier.
Measuring range: 1.5-5000 pg/mL.
Inter and intra assay CV: 1.7-8.5%.

23. Definitions of metabolic syndrome (MetS) used in the study:
   a) NCEP/ATP III criteria\textsuperscript{186}: Any three of the following five criteria.
      \begin{itemize}
        \item Waist circumference: $\geq 102$ cm in men and $\geq 88$ cm in women.
        \item Blood pressure $\geq 130/85$ mmHg.
      \end{itemize}
• Triglyceride concentration ≥ 150 mg/dL.
• HDL-C concentration ≤ 40 mg/dL in men and ≤ 50 mg/dL in women.
• Fasting plasma glucose ≥ 110 mg/dL.

b) South Asian modified NCEP Criteria for Asian Indians\(^\text{190}\) (SAM-NCEP): Any three of the following five criteria.

• Waist circumference ≥ 90 cm in men and ≥ 80 cm in women.
• Blood pressure ≥ 130/85 mmHg.
• Triglyceride concentration ≥ 150 mg/dL.
• HDL-C concentration ≤ 40 mg/dL in men and ≤ 50 mg/dL in women.
• Fasting plasma glucose ≥ 100 mg/dL.