METHODS & MATERIALS

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Chronic degenerative diseases such as obesity, diabetes, hypertension and coronary heart disease are making an important contribution to mortality over the past few years in the developing countries. Epidemiological transition coupled with modernization and industrialization has resulted in a rapid increase in the prevalence of these CDD. This has led to a change in the lifestyle of the people and these lifestyle related risk factors play an important role in the development of various CDD. Multiple risk factors often act synergistically causing a geometric increase in the overall risk of developing CDD. Identifying these risk factors is important and it has considerable health implications. Hence, the current study was carried out with an objective of analysing the various risk factors in the development of chronic degenerative diseases

This chapter deals with the various methods employed in carrying out the study. The methodology has been discussed in three sections as follows:

SECTION I

In this section the details regarding the general habits, dietary habits and the anthropometric measurements are discussed.

i) SELECTION OF SUBJECTS

The enrolment of the subjects was carried out from the training centre of the Indian Oil Corporation (IOC) in coordination with the IOC Hospital

(**Figure 10**) The study group comprised of all the employees and their spouses The subjects were asked to come to the training centre after an overnight fast

ii) GENERAL INFORMATION

General information in relation to the age, sex, marital status, economic background, and their general habits such as smoking, alcohol intake and exercise profile was collected

iii) ASSESSMENT OF NUTRITIONAL STATUS

Twenty-four hour dietary recall method was used for the assessment of nutritional status of the subjects The subjects were also asked regarding the frequency of consumption of non-vegetarian foods

iii) ANTHROPOMETRIC MEASUREMENTS

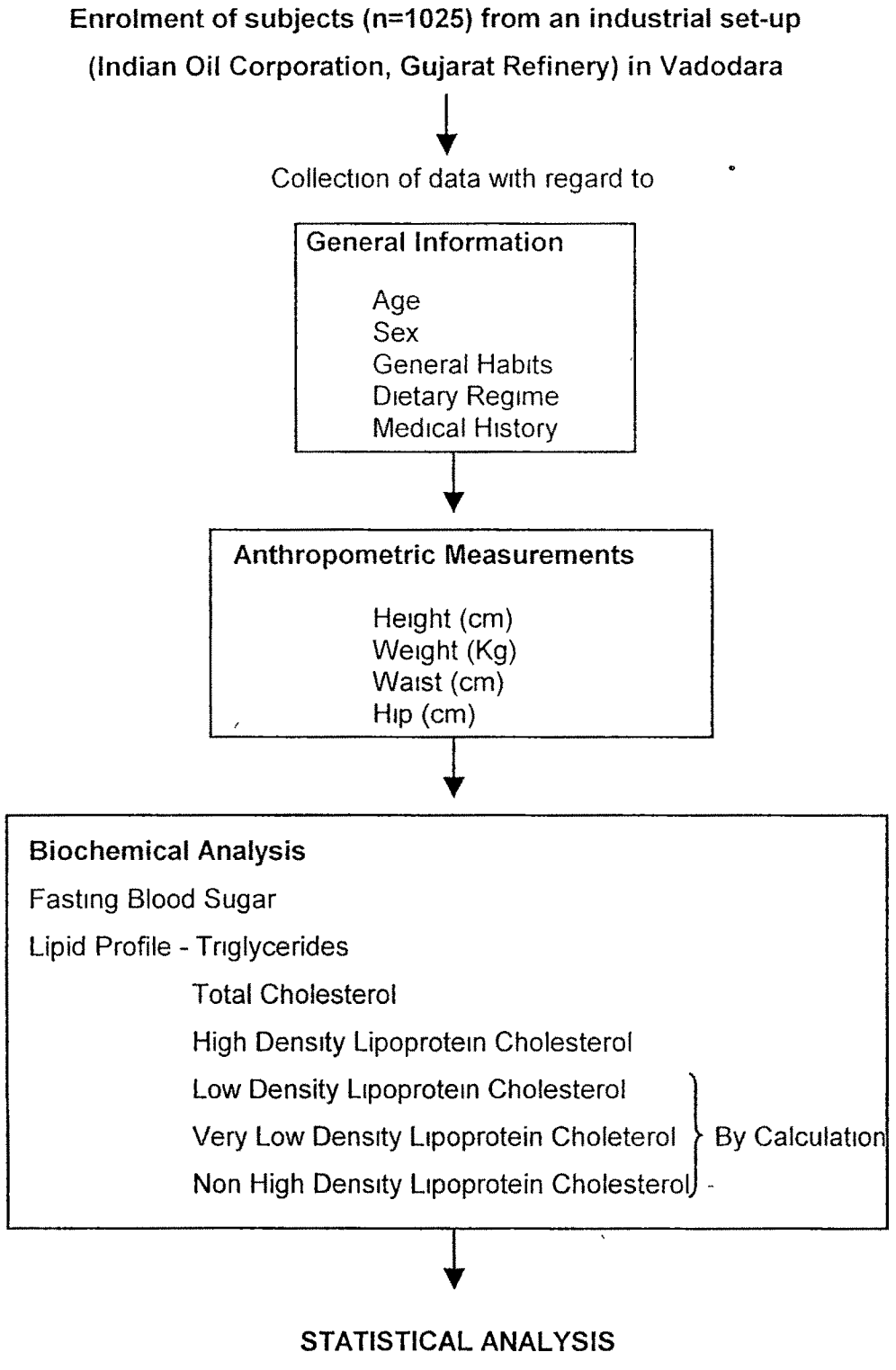
The standing height, weight, waist and hip, measurements were taken and the Body Mass Index (BMI) and the Waist Hip Ratio (WHR) ere calculated using the following formulas.

$$BMI = \frac{\text{Weight in Kg}}{\text{Height in}(m^2)}$$

$$WHR = \frac{\text{Waist Measurement (cm)}}{\text{Hip Measurement (cm)}}$$

FIGURE 10

Experimental Design
(SECTION I &II)



SECTION II

This section deals with the methodology for various biochemical estimations carried out Venous blood samples were collected after an overnight fast and the serum was separated, which was used for the estimation of various parameters like FBG, TC, TG and HDL-C The analysis was done using the Boehringer Mannheim enzymatic kits on a Boeheringer Mannheim Autotek instrument LDL-C and Very low density lipoprotein cholesterol (VLDL-C) were calculated using the Freidwalds formula (Friedwald et al, 1972) Non HDL-C was also calculated using a formula

1) ESTIMATION OF GLUCOSE (Enzymatic Colrimetric Method)

Reference The blood glucose was estimated using Boehringer Mannheim enzymatic kit (Germany)

Principle Glucose is oxidised by the enzyme Glucose Oxidase (GOD) to give gluconate and hydrogen peroxide Hydrogen peroxide in the presence of enzyme Peroxidase (POD) oxidizes phenol which combines with 4-aminophenazone to produce a red coloured 4-(p-benzoquinone-mono-imino) phenazone dye The intensity of the colour developed is proportional to the glucose concentration in the sample

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{4-(p-benzoquinone-mono-imino) phenazone} + 4\ H_2O$$

2) ESTIMATION OF TRIGLYCERIDES

Reference The estimation of triglycerides was done using Boehringer Mannheim enzymatic kit (Germany)

Principle Triglycerides in the sample is hydrolysed by lipase to glycerol and free fatty acids. Glycerol is phosphorylated by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate in a reaction catalyzed by glycerol-kinase (GK) Glycerol-3-phosphate is oxidised to dihydroxyphosphate in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GPO) In this reaction hydrogen peroxide is produced in equimolar concentration to the level of triglyceride present in the sample Hydrogen peroxide reacts with 4 aminophenazone and 4-chlorophenol in a reaction catalyzed by peroxidase The result of this oxidative coupling is 4-(p-benzoquinone-mono-imino) phenazone dye

$$\text{Triglycerides} + 3H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3\text{RCOOH}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + 4\ \text{aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{4-(p-benzoquinone-mono-imino)-phenazone} + 2H_2O + HCl$$

3) ESTIMATION OF CHOLESTEROL

Reference The estimation of cholesterol was done using Boehringer Mannheim enzymatic kit (Germany)

Principle Cholesterol esterase enzyme hydrolysis cholesterol ester to free cholesterol and fatty acid. Free cholesterol is oxidized by cholesterol oxidase to cholest-4-en-3 one and hydrogen peroxide Hydrogen peroxide formed reacts with 4-aminophenazne and phenol in the presence of peroxidase to produce pink coloured 4-(p-benzoquinone-mono-imino)-phenazone dye The intensity of the colour produced is proportional to the cholesterol concentration

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3 one} + H_2O_2$$

$$H_2O_2 + \text{4 aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{4-(p-benzoquinone-mono-imino)-phenazone} + 4H_2O$$

4) ESTIMATION OF HIGH DENSITY LIPOPROTEIN CHOLESTEROL

Reference The HDL-C was done using Boehringer Mannheim enzymatic kit (Germany)

Principle Chylomicrons, VLDL-C, and LDL-C are precipitated by adding phosphotungstic acid and magnesium ions to the sample Centrifugation

leaves only the HDL-C in the supernatant, their cholesterol content is determined enzymatically.

$$\text{Serum + Phoshotungstic acid + Mg ions} \longrightarrow \underset{\text{(LDL-C\&VLDL-C)}}{\text{Precipitate}} + \underset{\text{(HDL-C)}}{\text{Supernatant}}$$

$$\text{Cholesterol ester+ } H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol + RCOOH}$$

$$\text{Cholesterol } + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3 one + } H_2O_2$$

$$H_2O_2 + \text{4 aminophenazone + phenol} \xrightarrow{\text{peroxidase}} \text{4-(p-benzoquinone-mono-imino)-phenazone + } 4H_2O$$

5) CALCULATION OF LDL- CHOLESTEROL AND VLDL- CHOLESTEROL

Reference LDL-C and VLDL-C was calculated using Friedwalds formula (1972)

Calculation of LDL-C :

$$\text{LDL-C} = \text{Total Cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL-C}$$

Calculation of VLDL-C :

$$\text{VLDL-C} = \frac{\text{Triglycerides}}{5}$$

6) CALCULATION OF NON HDL- CHOLESTEROL

Non HDL-C was calculated using the following formula

Non HDL-C = Total Cholesterol – HDL-C

7) CRITERIA USED FOR THE ASSESSMENT OF VARIOUS CDD

The following criteria was used to diagnose various CDD in the industrial set up:

1) **Overweight/Obesity** The prevalence of overweight /obesity was found out by taking into consideration the BMI of the subjects using the WHO classification of obesity.

2) **Diabetes** Diabetes was diagnosed on the basis of the fasting blood glucose levels and the medical history

3) **Hypertension** The subjects were classified hypertensive based on the medical history of the subjects

4) **CHD** CHD subjects were diagnosed based on the past medical history of heart attack, stroke and those who had undergone bypass surgery, angioplasty etc.

5) **Hyperlipidemia / Hypercholesterolemia / Hypertriglyceridemia** The subjects wee classified as hyperlipidemic or hyper cholesterolemic or hypertriglyceridemic based on the total cholesterol and triglyceride levels.

8) STATISTICAL ANALYSIS

Students 't' test, Analysis of Variance, Correlation and Relative risk was done amongst various variables

SECTION III

In this section the methodology of the biochemical parameters studied on sub samples (Apolipoproteins and Total antioxidant assay) are discussed

Recent studies and reports indicate that additional biochemical markers such as the measurement of antioxidants and apolipoproteins are better markers for profiling the risk of non-communicable diseases apart form the customary use of the measurement of FBG, TC, TG, HDL-C etc Hence, normal, dyslipidemic, overweight or obese, diabetic and hypertensive subjects who were willing to participate were enrolled and analysis of the above mentioned parameters was carried out (**Figure 11**)

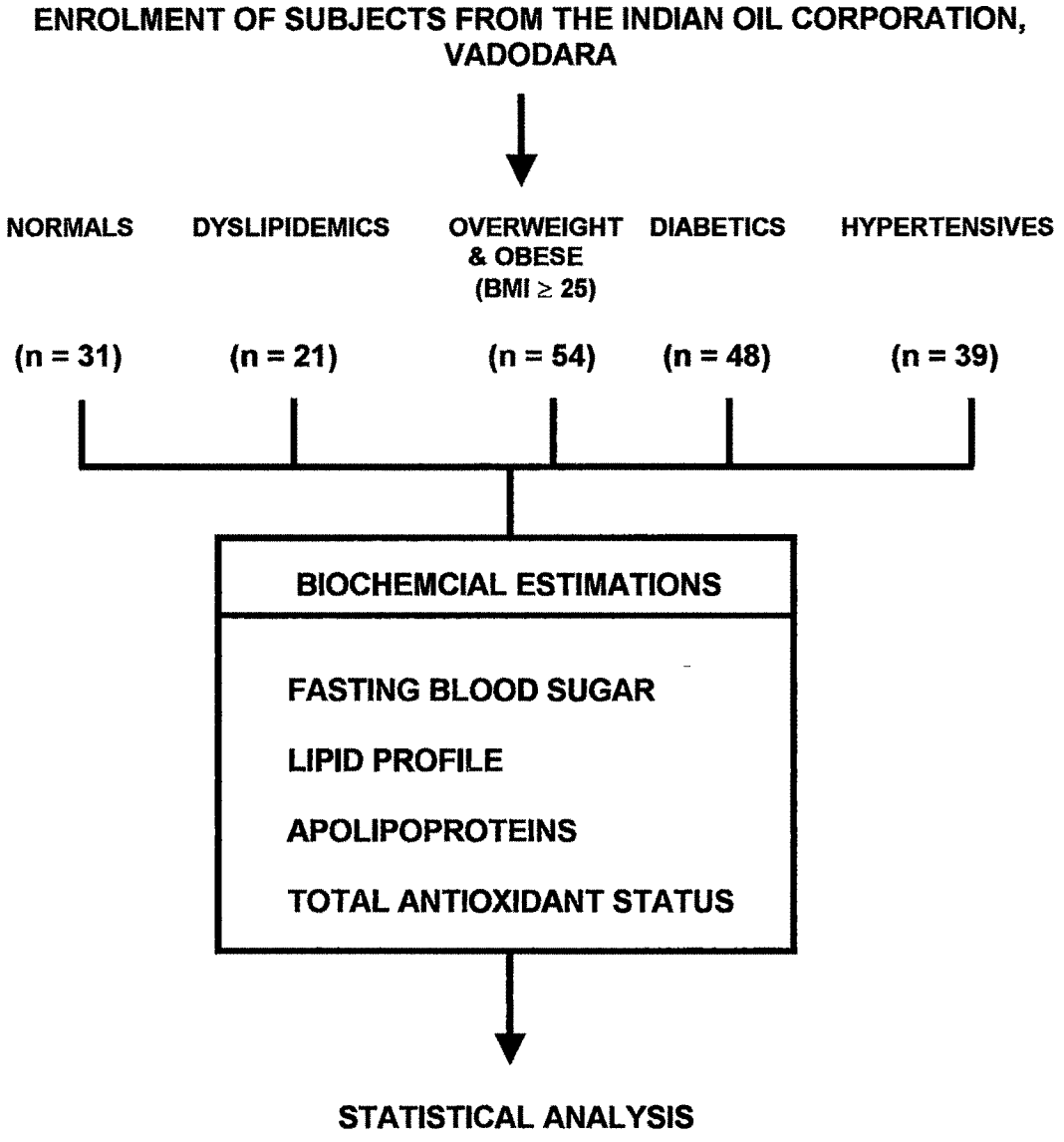
1) ESTIMATION OF APO A1 AND APO B

Reference Apolipoproteins were measured on the array protein systems (Beckman Instruments, Brea, California) following the principle of antigen antibody reaction by rate nephelometry.

Assay principle: The method employed in the Beckman APA or APB Test measures the rate of increase in light scattered from particles suspended in solution as a result of complexes formed during an antigen-antibody reaction In the performance of the APA/APB test, antibody of human apolipoprotein A-1/ apolipoprotein B is brought into contact with human apolipoprotein A-1/ apolipoprotein B in a sample The increase in light scatter from the antigen-antibody reaction is converted to a peak rate signal, which is a function of the

FIGURE 11

EXPERIMENTAL DESIGN
(SECTION III)



sample apolipoprotein A-1/apolipoprotein B concentration. Following calibration, the peak rate signal for a particular assay is automatically converted to concentration units by the analyzer

2) ESTIMATION OF TOTAL ANTIOXIDANTS

Reference The estimation of total antioxidant status was done using the Randox kit (Canada)

Assay principle ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation ABTS This has a relatively stable blue-green colour, which is measured at 60nm Antioxidants in the added sample cause suppression of this colour production to a degree, which is proportional to their concentration

$$HX - Fe''' + H_2O_2 \longrightarrow X - (Fe^{IV} = 0) + H_2O$$

$$ABTS + X - (Fe^{IV} = 0) \longrightarrow ABTS + HX - Fe'''$$

$HX - Fe'''$ = Metmyoglobin
$X - (Fe^{IV} = 0)$ = Ferrylmyoglobin
ABTS = 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]

3) Statistical Analysis

Students 't' test and regression was done between various variables

At a glance .....

SETION I

- Enrolment of one thousand and twenty five subjects from the industrial set-up

- The information on their socio-economic profile, clinical history, medical history, and general habits (smoking, chewing tobacco and alcohol consumption) were obtained through a structured questionnaire

- The nutritional status of the subjects was assessed using dietary history (24-hr dietary recall method ad food frequency method)

SECTION II

- Venous blood samples were collected after an overnight fast and the serum was separated, which was used for the estimation of various biochemical parameters

- Fasting blood sugar was estimated using an enzymatic kit.

- The estimation of lipid profile (TC, TG and HDL-C) of the subjects was also carried out with the help of enzymatic kits

- LDL-C and VLDL-C were calculated using the Friedwalds formula and Non HDL-C was also calculated by subtracting TC from HDL-C

SECTION III

- Recent studies and reports indicate that additional biochemical markers such as the measurement of antioxidants and apolipoproteins are better markers for profiling the risk of non-communicable diseases apart form the customary use of the measurement of FBG, TC, TG, HDL-C etc

- Apolipoproteins were measured on the array protein systems (Beckman Instruments, Brea, California) following the principle of antigen antibody reaction by rate nephelometry

- The estimation of total antioxidant status was done using the Randox kit (Canada)