MATERIAL & METHODS
Various "risk factors" that can increase the susceptibility of an individual to morbidity and mortality of CAD can broadly be classified under the heading of "risk markers".

This risk markers can further be subdivided into

(1) Non-modifiable
(2) Modifiable

Non-modifiable risk factors include age, sex (Male/ Female) and heredity which can not be influenced or changed by an interventional procedures or treatment schedule.

Modifiable risk factors include Diseased States such as hypertension and diabetes; Sex Hormonal changes such as menstruation, ovulatory or menopause phases and Life Style Choices such as diet, obesity, tobacco (smoking, chewing and as snuff), alcohol consumption etc.

The present work was conducted on about 250 females, who were made to fill a proforma (a copy of the same is enclosed in Appendix-I). After complete study of proforma indicating various modifiable and non modifiable factors, the females were placed in particular groups. Complete explanation of the research work with its importance was conveyed and the undertaking to co-operate as one of the subject in the study was taken from the females (a copy of the same is enclosed in Appendix-I).

The subjects were given certain instructions to be followed, which were necessary precautions for the study of Lipid Profile investigation for accuracy of the results. The precautions to be taken were as follows:
1. Subjects were advised to have normal diet and also perform normal activities which they have been doing earlier; but without gain or loss of weight.

2. Before the collection of blood they were advised to be on fasting for 12 hours i.e., without having any eatable items including, sugar, cream, coffee, tea, alcohol, medicine etc.

3. Subjects were advised to have normal water intake, but were asked not to take excessive water during the last 2-3 hours before blood collection.

**SAMPLE COLLECTION:**

On the day of collection, the blood was collected by the phlebotomy technique recommended by laboratory standardization panel (1988). In short the technique is as follows:

The subject was allowed to sit quietly for 15 minutes before collection of blood. The site of puncture was made sterile by an absorbent cotton soaked in methylated spirit. After cleaning the site, the liquid was allowed to be evaporated (not by blowing). Once the site was dried the tourniquet was applied 2 inches above the elbow joint, for more than 1-2 minutes. Then the blood was collected by venupuncture of the cubital vein by the use of sterile disposable needle (21"g) and 10 ml syringe.

After collection of the sample of the blood (approximately 7 ml) the site of puncture was again cleaned to remove blood sticking around. The cotton swab was pressed between the puncture with sufficient pressure so as to prevent oozing of blood.
The blood (approximately 7 ml), collected in the sterile disposable syringe is transferred for various biochemical estimations into the various bulbs as follows:

1. Fluoride bulb : 1 ml
2. Plain bulb : 6 ml

Blood collected in the fluoride bulb was centrifuged at 4000 rpm and the plasma was separated for estimation of glucose as explained below in the subheading, Biochemical Estimation (A). The blood collected in the plain bulb was allowed to clot and centrifuged at 4000 rpm serum was separated and was used for estimation of lipid profile and sex hormones on semi-autoanalyser SEACH-CH-100 Ames: marketed by Miles India Ltd., Baroda. The method is explained below in the sub-heading Biochemical Estimation (B & C). Serum of healthy control, obese and diabetic menopause subjects were used for estimation of apolipoprotein A-I (Apo A-I) and Apolipoprotein B (Apo B) on Turbitimer System (Turbitimer) manufactured by Behringwerke AG, Germany: marketed by E.Merck (India) Limited, Bombay. The method is explained below in subheading Biochemical Estimation (D).

BIOCHEMICAL ESTIMATIONS

A. Estimation of blood glucose:

Estimation of blood glucose was carried out by glucose oxidase peroxidase i.e., GOD-POD method (Trinder, 1969), with the help of "GLUCOZYM" reagent kit, marketed by Ortho Diagnostic System, Bombay.

Principle:

\[
\text{Glucose + } O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid + } H_2O_2
\]
Glucose is oxidized by glucose oxidase (GOD) to give gluconic acid and hydrogen peroxide. The hydrogen peroxide formed is broken down by peroxidase (POD) to water and oxygen. The latter oxidises phenol which on reacting with 4-aminophenazone gives a red coloured complex. The intensity of the red coloured complex is proportional to the concentration of glucose in the specimen under test, and is measured colorimetrically at 515 nm (range 500 to 530 nm). The colour remains stable for 2 hours at room temperature.

**Procedure:**

Clean, sterile, dry test tubes were taken and labelled as blank, standard and tests. The reagents were added as mentioned below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank(B)</th>
<th>Standard(S)</th>
<th>Tests(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucozyme working reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Plasma</td>
<td>-</td>
<td>-</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Standard (100 mg%)</td>
<td>-</td>
<td>0.01 ml</td>
<td>-</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.01 ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The contents of the tubes were thoroughly mixed, and placed in a water bath at 37°C for 15 minutes. The O.D. of the tests and the standard were measured against regent blank at 505 nm (range 500 to 530 nm). The results of glucose estimation were calculated as follows:

\[
\text{Glucose in mg%} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard}} \times 100
\]
B. Estimation of Lipid Profile:

The lipid Profile includes serum Total cholesterol (TC), Triglyceride (TG), HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, TC/HDL ratio and LDL/HDL ratio.

(i) Serum Total Cholesterol (TC):

Estimation of serum TC was carried out by enzymatic method (Allain, 1974), with the help of “Autopak” CHOLESTEROL reagent kit, marketed by Miles India Ltd. Baroda.

**Principle:**

\[
\begin{align*}
\text{Cholesteryl ester hydrolase} & \quad \text{Cholesteryl ester} \rightarrow \text{Cholesterol + FA} \\
\text{Cholesterol oxidase} & \quad \text{Cholesterol + O} \rightarrow \text{Cholest-4-en-3-one + H}_2\text{O}_2 \\
\text{Peroxidase} & \quad \text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminoantipyrine} \rightarrow \text{O-Quinoneimine (Red colour)}
\end{align*}
\]

It is a well established fact that cholesterol in the blood exists in two forms i.e., free (30%) and esterfied (70%). By the enzymatic method the esterfied cholesterol is hydrolysed by cholesteryl ester hydrolase to free cholesterol and fatty acid (FA). The free cholesterol produced and pre-existing in blood are oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase, oxidizes the chromogen (4-aminophenazone/phenol) to a O-quinoneimine; red coloured compound (Trinder reaction) which is read at 510 nm (range 500 to 530 nm) which remains stable for 2 hours if not exposed to direct light.
Procedure:

Clean, sterile, dry test tubes were taken and labelled as blank, standard and tests. The reagents were added as mentioned below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank(B)</th>
<th>Standard(S)</th>
<th>Tests(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol working reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard (200 mg%)</td>
<td></td>
<td>0.01 ml</td>
<td></td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.01 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The contents of the tubes were thoroughly mixed, and placed in a water bath at 37°C for 5 minutes. The O.D. of the test and the standard were measured against reagent blank at 505 nm. (range 500 to 530 nm). The results of total cholesterol estimation were calculated as follows:

\[
TC \text{ in mg\%} = \frac{O.D. \text{ of Test}}{O.D. \text{ of Standard}} \times 200
\]

(ii) Serum Triglyceride (TG):

Estimation of serum TG was carried out by enzymatic method. (McGowan et al., 1983), with the help of “Autopak” TRIGLYCERIDE reagent kit, marketed by Miles India, Ltd., Baroda.

Principle:

\[
\text{LP Lipase} \quad \text{Triglycerides} \quad \xrightarrow{\text{Glycerol + Free fatty acids.}} \quad \text{Glycerol Kinase} \quad \text{Glycerol + ATP} \quad \xrightarrow{\text{Glycerol-3-phosphate + ADP.}}
\]
Glycerophosphate Oxidase

Glycerol-3-phosphate + O₂ \[\rightarrow\] Dihydroxyacetone Phosphate + \(H₂O₂\)

Peroxidase

\(H₂O₂\) + 4-aminoantipyrine + 2-hydroxy-3, 5-dichlorobenzenesulfonate \[\rightarrow\] Quinoneimine dye + \(H₂O\) (Red colour)

Triglycerides incubated with lipoprotein lipase are hydrolysed to free fatty acids and glycerol. Conversion of glycerol takes place in the presence of ATP & glycerol kinase to glycerol-3-phosphate and ADP. The glycerol-3-phosphate is further oxidized to dihydroxy acetone phosphate and hydrogen peroxide by glycerol phosphate oxidase. \(H₂O₂\) so formed reacts with chromogens (2-hydroxy-3, 5-dichloro benzenesulfonate 4-aminoantipyrine) in presence of peroxidase to give quinoneimine dye a red coloured complex which is read at 510 nm (range 500 to 530 nm).

Procedure:

Clean, sterile, dry test tubes were taken and labelled as blank, standard and tests. The reagents were added as mentioned below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Tests (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG working reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Plasma</td>
<td>-</td>
<td>-</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Standard (200 mg%)</td>
<td>-</td>
<td>0.01 ml</td>
<td>-</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.01 ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The contents of the tubes were thoroughly mixed and placed at room temperature for 15 minutes. The O.D. of the tests and the standard were
measured against reagents blank at 505 nm (range 500 to 530 nm). The results of triglyceride estimation were calculated as follows:

\[
\text{TG in mg\%} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard}} \times 200
\]

(iii) Serum HDL cholesterol:

Estimation of serum HDL cholesterol was carried out by phosphotungstate method (Lopes - Virella et al., 1977), with the help of "Autopak" HDL-CHOLESTEROL reagent Kit marketed by Miles India Ltd., Baroda.

Principle:

The lipoproteins present in human serum are chylomicrons, VLDL, LDL & HDL. Chylomicrons, VLDL and LDL fractions were separated from HDL by precipitating HDL with phosphotungstic acid-magnesium chloride. After centrifugation, at 4000 rpm the HDL cholesterol fraction which remains in the supernatant is assayed with enzymatic cholesterol method as mentioned earlier in estimation of TC [B(i)].

Procedure:

The serum sample (0.2 ml) and precipitating reagent (0.2 ml) were pipetted into a centrifuge tube, mixed thoroughly and then centrifuged at 4000 rpm for 10 minutes. The clear supernatant was separated immediately and utilised for the HDL cholesterol estimation as mentioned below:
The contents of the tubes were thoroughly mixed and placed in a water bath at 37°C for 5 minutes. The O.D. of the tests and the standard were measured against reagent blank at 505 nm (range 500 to 530 nm). The results of HDL Cholesterol estimation were calculated as follows:

\[
\text{HDL in mg\%} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard}} \times 100^* 
\]

* Concentration of standard is 50 mg\% but for calculation purpose 100 mg\% is taken into account for the dilution of the serum sample because in the test sample along with the serum equal volume of precipitating reagent was also added, and thoroughly mixed. After centrifugation the supernatant is used for estimation.

(iv) VLDL & LDL Cholesterol:

The results of various estimations performed enzymatically as explained in (B) [i, ii & iii] were taken to calculate the concentration of VLDL and LDL cholesterol using Friedewald formula (Friedewald et al., 1972) as stated below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank(B)</th>
<th>Standard(S)</th>
<th>Tests(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol working reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Supernatant from above step</td>
<td>-</td>
<td>-</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>Standard (50 mg%)</td>
<td>-</td>
<td>0.02 ml</td>
<td>-</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.02 ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
VLDL in mg% = \frac{\text{TG}}{5}

LDL in mg% = \text{TC} - \text{VLDL} - \text{HDL}

(v) **Ratio calculation:**

TC/HDL and LDL/HDL ratios were calculated manually by using the results of estimations of different parameters as mentioned in (B) [i, ii, iii & iv].

C. **Estimations of Sex hormones:**

Estimation of Estradiol (E₂) and progesterone (P) hormones were carried out with the help of Estradiol EIA and Progesterone EIA bioMerieux kits marketed by Cadila Hospital Products Limited, Ahmedabad.

(i) **Serum Estradiol:**

The estimation of serum Estradiol (E₂) was carried out by quantitative enzyme immunoassay method of Ratcliffe and Col. (1988).
**PRINCIPLE**

The assay is based on a competitive reaction principle. After separating estradiol from its carrier proteins SBP (Sex Steroid Binding Protein) and albumin, the assay was performed in two steps as depicted below diagrammatically.

---

**immunological step**

- **1** Anti-estradiol coated tubes
- **2** Estradiol present in sample, standards and control

**enzymatic step**

- **3** Enzyme conjugate: horseradish peroxidase-labelled estradiol
- **4** Chromogen substrate: OrthoPhenylenediamine (O.P.D.)/H₂O₂

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**Incubation**

- 2 hours at 18-25°C
- 30 min at 18-25°C

**Washing**

**Stropping reagent**

- reading at 505 nm
- yellow-orange coloring
Procedure:

The enzyme immunoassay kit consistsof anti-estradiol tubes and EIA tubes. The anti-estradiol tubes were labelled as standards, control and samples. The various quantities of the reagents added were as tabulated below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Standards</th>
<th>In anti-Estradiol tubes</th>
<th>Control</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td>50 ul</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>50 ul</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>-</td>
<td>50 ul</td>
<td>-</td>
</tr>
<tr>
<td>Enzyme Conjugate Soln.</td>
<td>500 ul</td>
<td>500 ul</td>
<td>500ul</td>
<td>-</td>
</tr>
</tbody>
</table>

After addition of various reagents in the anti-estradiol tubes, the tubes were shaken on a Vortex mixer and incubated for 2 hours at 18-25°C in the dark. After incubation the liquid from each tube was aspirated rapidly (in less than 2 minutes) and 2ml of the working wash solution [sodium phosphate, sodium chloride, Tween 20 (sodium merthiolate)] was dispensed and again aspirated immediately. The washing procedure was repeated and all the traces of solution aspirated thoroughly in a series of no more than 48 tubes, and into it was added color working solution 500 ul [color 1 (Orthophenylene Diamine dihydrochloride) + color 2 (Sodium phosphate, Citric acid, H₂O₂)] in all tubes. After that the tubes were incubated without shaking for 30 minutes in the dark and the stopping reagent 2 ml [color 3 (1.8 N H₂SO₄)] was added and again shaken on a Vortex mixer. The optical density was read at 505 nm against the reagent blank. The estradiol concentration of each sample was determined using a calibration curve.

(ii) Serum Progesterone:

The estimation of serum Progesterone (P) was carried out by quantitative enzyme immunoassay method of Wood et al., (1985).
The assay is based on a competitive reaction principle. After liberating progesterone from its carrier proteins CBG (Corticosteroid Binding Globulin) and albumin using 8-Anilino-1-Naphthalene Sulfonic acid (ANS), the assay was performed in two steps as depicted below diagrammatically.

**immunological step**

1. Anti-progesterone coated tubes
2. Progesterone present in sample, standards and control
3. Incubation 2 hours at 18-25°C
4. Washing

**enzymatic step**

1. Incubation 30 min at 18-25°C
2. Stopping reagent
3. Reading at 505 nm
4. Yellow-orange coloring

**Enzyme conjugate:** horseradish peroxidase-labelled progesterone

**Chromogen substrate:** OrthoPhenylenediamine (O.P.D.)/H₂O₂
Procedure:

The enzyme immunoassay kit consists of anti-progesterone tubes and EIA tubes. The anti-progesterone tubes were labelled as standards, control and samples. The various reagents were added as tabulated below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>In anti-Progesterone tubes</th>
<th>In EIA tubes</th>
<th>Standards</th>
<th>Control</th>
<th>Samples</th>
<th>Reagent Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td>50 ul</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>50 ul</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>-</td>
<td>50 ul</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enzyme Conjugate Soln.</td>
<td>500 ul</td>
<td>500 ul</td>
<td>500 ul</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

After the addition of various reagents in the anti-progesterone tubes the tubes were shaken on a Vortex mixer and incubated for 2 hours at 18-25°C in dark. After incubation the liquid from each tube was aspirated rapidly (in less than 2 minutes) and 2 ml of the working wash solution [sodium phosphate, sodium chloride, Tween 20 (sodium merthiolate)] was dispensed and again aspirated immediately. The washing procedure was repeated and all the traces of solution aspirated thoroughly in a series of not more than 48 tubes, and into it was added color working solution 500 ul [color 1 (Orthophenylene Diamine dihydrochloride) + color 2 (Sodium phosphate, Citric acid, H₂O₂)] in all the tubes. After that the tubes were incubated without shaking for 30 minutes in dark and the stopping reagent 2 ml [color 3 (1.8 N H₂SO₄)] was added and again shaken on a Vortex mixer. The optical density was read at 505 nm against the reagent blank. The progesterone concentration of each sample was determined using a calibration curve.
D. Estimation of serum Apo A-I and Apo B:

Estimation of serum Apo A-I and Apo B were carried out with the help of Turbiquant reagents kits marketed by E. Merck (India) Limited, Bombay.

**Principle:**

The technique is based on immunochemical reaction. The apolipoproteins present in the human serum sample react with the specific antibodies present in the reagent to form immune complexes. The turbidity developed in the reaction due to formation of the immune complexes is measured photometrically. The concentrations are determined quantitatively by turbidimetric measurement of the maximum reaction velocity which is also known as peak-rate method. Simultaneously the maximum reaction velocity (Vmax) of precipitate formation and the time (tVmax) required to reach Vmax are measured by the Heidelberg curve (Metzmann, 1985).

The serum sample which was separated by centrifugation method as explained in sample collection, from which 50 ul of serum was taken in cuvette and diluted with 1000 ul of isotonic NaCl solution. This was done in a ratio of 1:21 as explained in the manual of the Turbitimer.

The cuvette contains the diluted serum sample was placed in the Turbitimer and then 500 ul of the Turbiquant reagent was added in to cuvette and then the instrument was allowed to measure the VMax and tVmax and determine the result by Heidelberg curve (Metzmann, 1985).

The result obtained by Heidelberg curve are evaluated by comparing the values obtained for the two reaction parameters with those of reference reagent determined at Behringwerke. The so-called “calibration field” is batch dependent and supplied with each pack in the form of a bar code. The results of Apo A-I and Apo B were calculated automatically and printed in g/L.
Protocol of Experiment:

I. Normal State:

Group 1: Control Healthy subjects.
(A) 18 to 35 years of age
(B) 36 to 50 years of age
(C) Above 50 years of age

II. Diseased State:

Group 2: Hypertensive subjects.
(A) 18 to 35 years of age
(B) 36 to 50 years of age
(C) Above 50 years of age

Group 3: Diabetic subjects.
(A) 18 to 35 years of age
(B) 36 to 50 years of age
(C) Above 50 years of age

III. Life Style Choices:

Group 4: Obese subjects.
(A) 18 to 35 years of age
(B) 36 to 50 years of age
(C) Above 50 years of age

Group 5: Snuff User subjects.
(A) 18 to 35 years of age
(B) 36 to 50 years of age
(C) Above 50 years of age
Statistical Methods:

The statistical analysis were carried out by comparing the results of various estimations in menstruating subjects (i.e., during menstruation and ovulatory phase) in various age groups and conditions in the same subjects by the use of students paired “t” test. Also statistical analysis was carried out in menstruating and menopause subjects in relation to non-modifiable and modifiable risk factors compared with their respective control subjects by the use of students unpaired “t” test, for obtaining the level of significance according to the method of Snedecor and Cochran (1967). The results are expressed in mg% (Glucose, TC, TG, HDL, LDL, VLDL) or in ng/l (Estradiol) or in ng/ml (Progesterone) and ratios (TC/HDL, LDL/HDL). The results are expressed as mean ± S.E.M.