CHAPTER II

MATERIALS AND METHODS
Materials

Serum samples were collected from Volunteers. Reduced glutathione (GSH), 5, 5’-Dithiobis2-nitrobenzoic acid (DTNB), Ethylene demine tetra acetic acid (EDTA), Riboflavin, Niro Blue Tetrazolium (NBT), Tris buffer, Bovin Serum, Ascorbic acid were from SRL. Vitemin E was from MERCK. Phenyl acetate was from Sigma Aldrich Company USA. Sulfuric Acid and all other chemicals were purchased from Ranbaxy chemicals (New Delhi). Acids, bases, solvents, and salts used for the purification were of analytical grade.

2.1. Subjects Selections

The subjects from Iran were recruited from among individuals who visited Pasteur Laboratories at Tonekabon Iran. The Indian subjects were selected from among those who visited Biochem Diagnostic and research Laboratories Mysore India.

2.1.1. Comparison of Iranians in India with age and sex matched Indians

Iranians were chosen who age is between 25-60 years and who have lived in India for at least 1 year. Age, with accuracy of ± 1year, and sex matched Indians were chosen from among the volunteers for the present study. 30 Iranians and 30 Indians were taken; 24 male and 6 female Iranian as well as Indian.

The study protocol was approved by ethical committee of Mysore University, India and the consent was taken from all of the subjects (Iranians and Indians). (Informed consent form in Annexure 1.

2.2. Data Collection:

General demographic characteristics (LifeStyle) and dietary intake were collected of all the samples.

2.2.1. General characteristics:

The weight of subjects (Iranians and Indians) were recorded by a weight machine with accuracy of ± 100 gr, models KRPUUS (NewDelhi, India) and standing height measured without shoes to the nearest 0.5 centimeter with use of commercial meter, with the shoulders in relaxed position and arms hanging freely. Body mass
index (BMI) calculated by weight (kg) divided by height in meter square (m²). Blood pressure was monitored via a sphygmomanometer (Diamond, Mercurial, India) and Stetitoscope (Life Line, India) in relaxed position. Other information: age, sex, education level, profession, family income, and lifestyle habits (exercise, stress) and food habits (Recall method, types of foods, oils used, spices, fresh vs cooked – pressure cooked, boiled, roasted, fried, etc). Other health problems (medical history) were collected via interview of the subjects (Annexure 1).

2.3. Blood analysis:

5ml of blood was drawn from subjects (Iranians and Indians) in fasting and aliquoted in two tubes without anticoagulant at Biochem Diagnostic and Research Lab, Mysuru, India. One of that was used for lipid profile in Biochem Lab by enzymatic method using commercial kit. another tube was used in the determination of antioxidant statuses (GSH, Total antioxidant capacity by ferricyanide reduction and phospho molybdate reduction, and Reduced Glutathione) and susceptibility of LDL to oxidation. Serum was sepreated by centrifugation (REMI, R8C LABORATORY CENTRIFUGE, India) at 3000 rpm for 10 min and serum was aspirated and stored at -20 degrees until used.

2.3.1. Biochemical analysis

Serum was analyzed for various biochemical parameters by enzymatic methods using commercial kit [TG & Cholesterol (ANAMOL LABORATORIES PVT. LTD. Maharashtra, India), LDL-C & HDL-C (Accurex Biomedical Pvt.Ltd. Mumbai, India), C-Reactive Protein (HS-CRP) (FUTURA SYSTEM S.R.L. India.), MINDRAY CHEMISTRY ANALYZER, BS-200 (CHINA)].

Total Cholesterol (TC), Triglyceride (TG) and HDL – Cholesterol were assayed using commercial kits. TG and Cholesterol kits were from ANAMOL LABORATORIES PVT. LTD. Maharashtra, India, HDL-C kit was from Accurex Biomedical Pvt. Ltd. Mumbai, India. C-Reactive Protein (HS-CRP) (FUTURA SYSTEM S.R.L. India.), MINDRAY CHEMISTRY ANALYZER, BS-200 (CHINA). TG, Cholesterol and HDL kits were also from PARS AZMOON Pvt. Ltd. Karaj, Iran.
2.3.1.1. Cholesterol

**Principle**

Cholesterol esters + H₂O → **Cholesterol esterase (CHE)** → Cholesterol + Fatty Acids

Cholesterol + O₂ → **Cholesterol oxidase (CHO)** → Cholestenone + H₂O₂

H₂O₂+4-Aminoantipyrine + phenol(Cromogen) → **Peroxidase(POD)** → Quinoneimine dye(Red Dye)+ H₂O

**Reagents**

- Buffer PH=7.5 100 mmol/L
- Cholesterol Oxidase > 10 IU/L
- Cholesterol Esterase > 135 IU/L
- Peroxidase > 495 IU/L
- Chromogen 0.5 mmol/L

**Sample**: serum

**Procedure**

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Sample/Standard</th>
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</thead>
<tbody>
<tr>
<td>Sample/Standard</td>
<td>10μl</td>
<td>10μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10μl</td>
<td>10μl</td>
</tr>
<tr>
<td>Reagent</td>
<td>1000μl</td>
<td>1000μl</td>
</tr>
</tbody>
</table>

After mixing were incubated for 5 min at 37°C and then the absorbance was read against blank at 546nm.

**Calculation**:  
Total Cholesterol (mg/dl) = Abs.of Sample/ Abs.of Standard × 200

**Normal Values (Reference range) and the Report format**

- Normal < 200 mg/dl
- Borderline 200-239 mg/dl
- High > 240 mg/dl
2.3.1.2. High-density lipoprotein cholesterol (HDL-C) (Gotto, 1988)

**Principle**

**Reaction 1**

\[ \text{LDL, VLDL, CM} \xrightarrow{\text{detergent1}} \text{miscellary cholesterol} \xrightarrow{\text{CHE & CHO}} \text{H}_2\text{O}_2 \]

\[ \text{H}_2\text{O}_2 + \text{DSBmt} \xrightarrow{\text{POD}} \text{colourless product} \]

\[ \text{HDL} \xrightarrow{\text{detergent1}} \text{HDL} \]

**Reaction 2**

\[ \text{HDL} \xrightarrow{\text{detergent2}} \text{miscellary cholesterol} \xrightarrow{\text{CHE & CHO}} \text{H}_2\text{O}_2 \]

\[ \text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine +DSBmt} \xrightarrow{\text{POD}} \text{coloured product} \]

**Reagent 1**

- N,N-bis (4-sulfobutyle)-m-toluidine disodium salt (DSBmt) 0.5 mmol/l
- Cholesterol oxidase 1 IU/ml
- Peroxidase
- Detergent 1
  - Good, s buffer solution; PH 6.0

**Reagent 2**

- 4-Aminoantipyrine 1 mmol/l
- Cholesterol esteras
- Detergent 2
  - Good, s buffer solution; PH 6.0

**Procedure**

<table>
<thead>
<tr>
<th>Time</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Sample: 3µl</td>
</tr>
<tr>
<td>18</td>
<td>R2 : 100 µl</td>
</tr>
<tr>
<td>3mi</td>
<td>R1 : 300 µl</td>
</tr>
<tr>
<td>17</td>
<td>A1 at</td>
</tr>
<tr>
<td></td>
<td>A2 at</td>
</tr>
</tbody>
</table>
Assay Point: 0, 17, 35
Linear Range: 1.5 to 150mg/dl

Calculation
Fully automated systems automatically calculate the HDL-C concentration of each sample.
Result in mmol/l = Result in mg/dl × 0.0259

Normal Values (Reference range) and the Report format
Men: 30-70 mg/dl
Woman: 30-85 mg/dl

2.3.1.3. Low-density lipoprotein cholesterol (LDL-C) (Center for Disease control/NIH manual 1988)

Principle

Reaction 1

\[
\text{LDL} \rightarrow \text{detergent1} \rightarrow \text{miscellary cholesterol} \rightarrow \text{CHE & CHO} \rightarrow \text{H}_{2}\text{O}_{2} \\
\text{CM} \rightarrow \text{H}_{2}\text{O}_{2} + \text{4-Aminoantipyrine} \rightarrow \text{POD} \rightarrow \text{colourless product} \\
\text{LDL} \rightarrow \text{detergent1} \rightarrow \text{LDL} \\
\]

Reaction 2

\[
\text{LDL} \rightarrow \text{detergent2} \rightarrow \text{miscellary cholesterol} \rightarrow \text{CHE & CHO} \rightarrow \text{H}_{2}\text{O}_{2} \\
\text{H}_{2}\text{O}_{2} + \text{4-Aminoantipyrine +DSBmt} \rightarrow \text{POD} \rightarrow \text{coloured product} \\
\]

Reagent 1
Detergent 1
4-Aminoantipyrine 0.5mmol/l
Cholesterol oxidase 1.2 U/ml
Cholesterol esterase
Peroxidase
Good's buffer solution; PH 6.3
Reagent 2
Detergent 2
N,N-bis (4-sulfobutyle)-m-toluidine disodium salt (DSBmt) 1.0 mmol/l
Good’s buffer solution; PH 6.3

Procedure

<table>
<thead>
<tr>
<th>0</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>3mi</td>
</tr>
<tr>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Sample: 3µl
R1 : 300 µl
R2 : 100 µl
Assay Point: 0, 17, 35
Linearity Range: 1.5 to 450mg/dl

Calculation
Fully automated systems automatically calculate the LDL-C concentration of each sample.
Result in mmol/l = Result in mg/dl × 0.02586

Normal Values (Reference range) and the Report format
The following NCEP cutpoints for patient classification for the prevention and Management of coronary heart disease:

<table>
<thead>
<tr>
<th>Classification</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desirable</td>
<td>&lt;130mg/dl(&lt;3.36mmol/l)</td>
</tr>
<tr>
<td>Borderline High Risk</td>
<td>130-159mg/dl(3.36-4.11mmol/l)</td>
</tr>
<tr>
<td>High Risk</td>
<td>&gt;160mg/dl(4.14mmol/l)</td>
</tr>
</tbody>
</table>
2.3.1.4. Triglyceride

**Principal**

Triglyceride $\xrightarrow{\text{Lipoprotein Lipase (LPL)}}$ Glycerol + Fatty acid

Glycerol + ATP $\xrightarrow{\text{Glycerol kinase (GK)}}$ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O$_2$ $\xrightarrow{\text{Glycerol-3-phosphate oxidase (GPO)}}$ Dihydroxyacetone phosphate + H$_2$O$_2$

H$_2$O$_2$ + Phenolic chromogen $\xrightarrow{\text{Peroxidase (POD)}}$ Red compound

**Reagent**

Buffer, PH 6.8 50 mmol/l

Lipase $> 2000$ IU/L

Glycerol kinase 300 IU/L

Glycerol phosphate oxidase Peroxidase 1000 IU/L

ATP 1.0 mmol/l

Chromogens 2.0 mmol/l

**Sample:** serum

**Procedure**

<table>
<thead>
<tr>
<th>Blank</th>
<th>Sample/Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample/Standard</td>
<td>10µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10µl</td>
</tr>
<tr>
<td>Reagent</td>
<td>1000µl</td>
</tr>
</tbody>
</table>

After mixing were incubated for 10 min at 37°C and then the absorbance was read against blank at 546nm.

**Calculation:**

Triglyceride (mg/dl) = Abs.of Sample/ Abs.of Standard $\times 200$
Normal Values (Reference range) and the Report format

Normal          40-150mg/dl
Borderline     > 150 mg/dl
Abnormal     > 240 mg/dl

2.3.1.5. Human Serum C-Reactive Protein(HS-CRP)

“C reactive protein HS” is a quantitative turbid metric test ultrasensitive for the measurement of low levels of C-reactive protein (CRP) in human serum.

Reagents

1. Deluent
   Tris Buffer   PH 8.2                           20 mmol/l
   Sodium Azide                 0.95 g/l

2. CRP-Latex        (1×5 ml)
   Latex particles coated with IgG anti-human CRP   PH=7.3
   Sodium Azide                 0.95 g/l

3. Calibrator        (1×2 ml)
   Human serum                          value stated
   on vial label
   Sodium Azide                 0.95 g/l

Preparation & stability of working solution

Sodium Azide                 0.95 g/l
Diluent                        liquid & ready to use
CRP-Latex                     liquid & ready to use
Calibrator                    liquid & ready to use

One reagent procedure

Gently swirl the latex before use & prepare the necessary amount of working solution mixing 9ml of diluents with 1ml of latex.

Two reagent procedure

Reagents ready to use

Sample: serum
Procedure

<table>
<thead>
<tr>
<th>One reagent procedure</th>
<th>Two reagent procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample/Calibrator</td>
<td>Sample/Calibrator</td>
</tr>
<tr>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>workingSolution</td>
<td>Diluents</td>
</tr>
<tr>
<td>1000 µl</td>
<td>900 µl</td>
</tr>
<tr>
<td>CRP Latex</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

After mixing were read the first absorbance of calibrator & samples (A1) and then were incubated for 4 min at 37°C and then the second absorbance of calibrator & samples (A2) were read against blank at 546 nm.

Calculation

\[
\text{CRP (mg/l)} = \frac{(A2-A1) \text{ sample}}{(A2-A1) \text{ calibrator}} \times \text{ calibrator value}
\]

Normal Values (Reference range) and the Report format

Below 3 mg/L

PON1 (Aryl esterase) activity

Aryl esterase activity was calculated from initial velocity plot Aryl esterase activity was determined by the method of Gan et al (1991). Briefly the cuvette contained 2 mM phenyl acetate in PON buffer (10 mM Tris HCl pH8.00 and 200 mM CaCl2) in a total volume of 1.0 ml. The reaction was initiated by the addition of enzyme [either serum for (1:100) diluted or HDL (1:50) diluted with PON buffer]. An increase in absorbance at 270 nm for up to 3 min was recorded at intervals of 1 min. Blanks without enzyme were used to correct for spontaneous hydrolysis of phenyl acetate. DOD was calculated for the linear part of the curve. One unit of enzyme activity was defined as amount of enzyme that released 1 µmol of phenol /minute under these conditions, the molar extinction coefficient of phenol 1310 M⁻¹ cm⁻¹ was used to calculate the product formed.

Determination of total antioxidant activity

For total antioxidant assay various concentrations (5, 10 and 20 µg in 1 ml) of Standard Ascorbic acid were mixed with 1 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated in a water bath at 95°C for 90 min. After cooling to room
temperature, the absorbance was measured at 695 nm. 900 µl of serum was used as unknown in the above assay and the total antioxidant capacity was calculated in comparison with ascorbic acid.

**Determination of Reduced Glutathione (GSH)**

Reduced glutathione in the serum was assayed using the reaction with DTNB and compared with a standard solution of reduced glutathione [8]. Serum (0.2 ml) was mixed with EDTA solution (1.8 ml, 0.1% w/v) and centrifuged to remove any precipitate. To the supernatant, DTNB reagent (1 ml, 40 mg, 5,5' dithiobiobis-2 nitro benzoic acid in 100 ml of 1% (w/v) sodium citrate) was added and the absorbance was measured at 420 nm. A calibration curve was prepared using reduced glutathione (0 to 60 µg).

**Isolation of Human LDL**

LDL and HDL were prepared from either pooled serum or individual serum samples by density gradient centrifugation carried out according to the method of Redgrave et al. (1975). A stepwise gradient was prepared as follows: 10 ml of serum was mixed with solid KBr such that the density was about 1.3 g/ml. 30 ml of saline, pH7, was layered on top of the serum sample. The tubes were centrifuged in a Sorvall ultra centrifuge in F-50L fixed angle rotor for 3 hours, at 40000 rpm (200 000 g) at 4°C. After centrifugation, the tubes were carefully removed from the rotor and placed in the vertical position. The fractions were aspirated from the top. The LDL fraction appears as a yellow layer in the center of the tube, and HDL appears as an orange-layer at the bottom of the tube. The LDL and HDL fraction were collected separately. LDL and HDL containing fractions were dialyzed in the dark for 6 and 8 hours against double distilled water.

**Determination of Lipoprotein Oxidation**

Lipid oxidation in lipoproteins was assessed by spectrophotometric monitoring of conjugated diene formation, according to the method of Esterbaure et al. (1992) with slight modification. Briefly, serum (5 µl) was diluted with phosphate buffered saline (PBS, 995 µl) (5 mM phosphate buffer, 125 mM NaCl, pH 7.4). LDL and HDL were diluted 1:50 with PBS. 100 µl of diluted
serum sample, LDL or HDL was mixed with 850µl PBS and 50µl of 5µ Mcopper sulfate (CuSo4). The OD of the mixture was monitored at 234 nm every 10 min for up to 90min. The optical density was plotted against time. From this plot the duration of the lag time, time of completion of oxidation and time of decline phase were determined.

The serum, LDL and HDL were also oxidized by Benzoxyl peroxide. Benzoxyl peroxide (0.1 gr) was dissolved in 1ml Chloroform. Then 5 µl of this was transferred into test tubes and the solvent was removed. To the tubes 895µl PBS was added and vigorously vortexed to suspend the benzoxyl peroxide. Diluted serum sample LDL or HDL (100µl) were then added and the absorbance of the mixture was measured at 234 nm every 10 min for up to 90min. The optical density was plotted against time. From this plot the duration of the lag time, time of completion of oxidation and time of decline phase were measured.

**Determination of protection of lipoprotein from oxidation by Ascorbic acid**

Protection of serum lipoproteins from oxidation by Ascorbic acid was assessed by spectrophotometric monitoring of the formation of conjugated dienes by copper induced oxidation as described above. However prior to inducing oxidation by copper sulphate, the serum sample was mixed with increasing concentration of ascorbic acid (0.6 to 5.7 µM). The reaction was monitored by for up to 90min at 234 nm.

**Determination of protection of lipoprotein from oxidation by Vitamin E**

Protection of serum lipoproteins from oxidation by Vitamin E was assessed by spectrophotometric monitoring of the formation of conjugated dienes by copper induced oxidation as described above. However prior to inducing oxidation by copper sulphate, the serum sample was mixed with increasing concentration of Vitamin E (0.2 to 2 µM). The reaction was monitored by for up to 90min at 234 nm.

**Protection of PON activity from oxidation by Ascorbic acid and Vitamin E:**

Protection of serum PON activity from oxidation by Ascorbic acid or Vitamin E was assessed by spectrophotometric monitoring of the PON activity using phenyl acetate as substrate by copper induced oxidation as described above. However
prior to inducing oxidation by copper sulphate the serum sample was mixed with increasing concentration of ascorbic acid (0.6 to 5.7 µM) or vitamin E (0.2 to 2 µM). 234 nm absorbance was monitored for 90 min. The PON activity was determined at the end of 90 min as described above.

**Determination of LDL oxidation by modified thiobarburic acid (TBA) assay:**

LDL (100 µl) was oxidized by 50 µl of Cu++ (5 µM copper at 25 °C, pH 7.4) were incubated at 37°C in Different time (0, 30, 60 min) then added 1.5 ml of 20% (v/v) acetic acid, 200 µl of 8.00% (w/v) sodium dodecyl sulfate (SDS); and To this were added 1.5 ml of fresh 0.8% (w/v) TBA. The tubes were vortexed well. The tubes were covered with a glass marble and heated at 95°C for 15 min. The samples were then cooled to room temperature. Three milliliters of n-butanol was added to each tube, the tubes were vigorously mixed again and centrifuged for 15 min at approximately 2500rpm, and the organic phase was carefully removed with a pipette and transferred to a new set of glass tubes. The absorbance of the samples were measured at 532 nm.

**Estimation of protein**

The protein concentration of serum LDL and HDL were measured by the methods of Lowry using bovine serum albumin (BSA) as a standard. For this method HDL was diluted with phosphate buffered saline [PBS (5 mM phosphate buffer, 125mM NaCl, pH 7.4)].

**Statistical analysis**

Mean and standard deviation were calculated for the samples. Comparison between two groups was done using Students t test. Correlation coefficients were calculated by Pearsons Product-Moment correlation.

PERT chart was drawn for qualitative data using Microsoft Excel graphics.