The study was carried out in the Department of Biochemistry GR Medical College, Gwalior. The study was done on 500 individuals, which included 400 Non Alcoholic Fatty Liver type II diabetic obese and 100 healthy individuals. Age matched healthy control subjects were selected from known families. The written consent of patients was also taken before starting the study. A record of clinical history and previous investigations of patients disorders were compiled in a Proforma (Proforma enclosed).

A proforma containing the relevant findings of clinical, biochemical and physiological investigations were recorded on preset questionnaire as base line record. All ethical measures were taken prior and during the study. 5 ml of blood sample was withdrawn from the anticubital vein following overnight fasting. The blood sample was collected in plain, fluoride and EDTA vacutainers. The blood sample was analyzed for biochemical investigations which include:

1. Fasting Blood Sugar (FBS)
2. Total Cholesterol (TC)
3. Triglyceride (TG)
4. High density-lipoprotein cholesterol (HDL-c)
5. Low density lipoprotein cholesterol (LDL-c)
6. Very low density lipoprotein cholesterol (VLDL-c)
7. Total bilirubin
8. Serum glutamate pyruvate transaminase (SGPT)
9. Alkaline phosphatase (ALP)
10. Gamma- Glutamine transpeptidase (GGT)
11. Fasting Insulin
12. Insulin resistance by HOMA –IR calculation method
13. high sensitive C-Reactive Protein (hs-CRP)

**Separation of serum:**

Blood sample was collected in plain vial and incubated at 37°C for 30 minutes. After incubation, clot was removed and remaining sample was taken in centrifuge test tube. Samples were centrifuged at 3000 rpm for 10 to 20 minutes. Supernatant collected in clean and dry serum test tube for analysis of fasting blood glucose, lipid profile and insulin as well as inflammatory markers hs-CRP and Liver enzyme.

**Biochemical Investigations:**

Biochemical parameters, fasting blood sugar and lipid profile were estimated by fully automated analyser in a commercially available kit (BioSystems S.A., Barcelona, Spain) according to manufacturer instructions.
ESTIMATION OF SERUM GLUCOSE

METHOD: (Enzymatic-colorimetric-Trinder-End Point).

PRINCIPLE

Glucose oxidase (GOD) oxidizes glucose to gluconic acid & hydrogen peroxide. In presence of enzyme peroxides, released hydrogen peroxide is coupled with phenol & 4-Aminoantipyrine (4-AAP) to from colored quinoneimine dye. Absorbance of colored dye was measured at 505 nm & is directly proportional to glucose in the sample.

\[
\text{Glucose oxidase} \\
\text{Glucose + O}_2 + \text{H}_2\text{O} \rightarrow \text{Gluconic acid + H}_2\text{O}_2
\]

\[
\text{Peroxidase} \\
\text{H}_2\text{O}_2 + \text{Phenol + 4-AAP} \rightarrow \text{Quinoneimine dye + H}_2\text{O}
\]

Reagent composition

- Phosphate buffer, pH 7.4: 13.8 mmol/L
- Phenol: 10 mmol/L
- Glucose oxidase: > 10000 mmol/L
- Peroxidase: >700 U/L
- 4-Aminoantipyrine: 0.3 mmol/L
Sample: Serum free of haemolysis. Serum is stable 8 hrs. At 25°C and up to 3 days at 2-8°C.

PROCEDURE

<table>
<thead>
<tr>
<th></th>
<th>BLANK</th>
<th>CALIBRATION</th>
<th>TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>REAGENT-R</td>
<td>300 µl</td>
<td>300 µl</td>
<td>300 µl</td>
</tr>
<tr>
<td>DISTILLED WATER</td>
<td>3 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CALIBRATER</td>
<td>-</td>
<td>3 µl</td>
<td>-</td>
</tr>
<tr>
<td>SAMPLE</td>
<td>-</td>
<td>-</td>
<td>3 µl</td>
</tr>
</tbody>
</table>

Mix and after 11 minutes and 30 seconds of incubation read the absorbance (A)

Wavelength 505 nm, Temperature 37°C

Read against reagent blank

Calculation

\[
\frac{A_{\text{sample}}}{A_{\text{calibrator}}} \times n = \text{calibrator factor}
\]

Conversion factor

\[
\text{mg/dl} \times 0.0555 = \text{mmol/L}
\]

\[
\text{mg/dl} \times 0.01 = \text{g/L}
\]

Reference Values

70-105 mg/dl (3.89-5.83 mmol/L).
ESTIMATION OF SERUM TOTAL CHOLESTEROL

METHODE: (Enzymatic-Colorimetric -Trinder- End point).

PRINCIPLE

Enzymatic colorimetric determination of total cholesterol according to the following reactions:

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{Chole Esterase}} \text{Cholesterol} + \text{fatty acid}
\]

\[
\text{Cholesterol} + \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{Chol. Oxidase}} \text{Cholesteone} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-AAP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\text{H}_2\text{O}
\]

Reagent composition

- Sodium cholate: 5 mmol/L
- Phenol: 24 mmol/L
- Cholesterol esterase: > 180 U/L
- Cholesterol oxidase: > 200 U/L
- Peroxidase: > 1000 U/L
- 4-Aminoantipyrine: 0.5 mmol/L, pH 7.0
Cholesterol Standard 200 mg/dl

**Reagent Preparation:** Reagent provided is ready to use.

Stability of reagents: To store 2-8°C and protected from light.

**Samples:** Heparin or EDTA plasma from fasting patients.

**Storage:** Samples are stable 5 to 7 days if stored at 4°C, 3 months at -20°C and several years at -70°C.

**PROCEDURE**

<table>
<thead>
<tr>
<th></th>
<th>BLANK</th>
<th>STANDARD</th>
<th>SAMPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent -R</td>
<td>1000µl</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Standard</td>
<td>------</td>
<td>10µl</td>
<td>------</td>
</tr>
<tr>
<td>Sample</td>
<td>------</td>
<td>------</td>
<td>10µl</td>
</tr>
</tbody>
</table>

Mix and incubate for 5 min. at 37°C. Read the absorbance of STD. And sample against reagent blank.

Wavelength : 505 nm

Temperature : 37°C

Read against reagent blank.

**CALCULATION**

\[
A_{\text{Sample}} / A_{\text{Standard}} \times n = n \text{ = standard concentration}
\]

**REFERENCE VALUES:** < 200 mg/dl
ESTIMATION OF SERUM TRIGLYCERIDES

METHOD: (Enzymatic-colorimetric end point method)

PRINCIPLE

Triglycerides in the sample originate, by means of the coupled reactions described below, a coloured complex that can be measured by spectrophotometer.

\[
\text{Triglycerides} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-P} + \text{ADP}
\]

\[
\text{Glycerol-3-P} + O_2 \xrightarrow{\text{G-3-P-oxidase}} \text{Dihydroxyacetone-P} + H_2O_2
\]

\[
2H_2O_2 + 4\text{-AAP} + 4\text{-Chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O_2
\]

Reagent composition

- Potassium ferrocyanide: 10 µmol/L
- Amino-4-antipyrine: 0.31 mmol/L
- Lipoprotein lipase: 2000 U/L
Glycerol Kinase 500 U/L
Glycerol-3- phosphate Oxidase 4000 U/L
Peroxides 500 U/L

**Standard**

Glycerol (triglycerides equivalent) 200 mg/ dl

**Stability of reagents**

To store at 2-8°C and protected from light.

**samples**

Serum or plasma from fasting patients. Collects the samples in EDTA tubes and stops free of glycerol. Sample are stable 5 to 7 days if stored at 2-8°C, 3 months at -15 to 20°C and several years at -70°C.

**PROCEDURE**

This reagent can be used on most analysers, semi automated analysers and Manual methods. The applications are available on request.

| Wavelength | 505 nm |
| Temperature | 37°C |

Read against reagent blank.

<table>
<thead>
<tr>
<th></th>
<th>BLANK</th>
<th>STANDARD</th>
<th>SAMPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent R</td>
<td>1000µl</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Standard</td>
<td>-------</td>
<td>10µl</td>
<td>-------</td>
</tr>
<tr>
<td>Sample</td>
<td>-------</td>
<td>-------</td>
<td>10µl</td>
</tr>
</tbody>
</table>
Mix and incubate for 10 min at 37°C. Read the absorbance’s of std. (A)

After a 425 seconds incubation.

**CALCULATION**

\[
\frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times n = \text{Standard Concentration}
\]

**REFERENCE VALUES:** up to 150 mg/dl.

**ESTIMATION OF HDL-CHOLESTEROL**

**METHOD:** PEG- CHOD- PAP, end point assay with lipid clearing factor (LCF).

Low density lipoprotein (LDL) Cholesterol, very low density lipoprotein (VLDL) Cholesterol and chylomicron fractions are precipitated by addition of polyethylene glycol 6000(PEG). After centrifugation, the high Density lipoprotein (HDL) fraction remains in the supernatant is determined with CHOD-PAP method.

**Reagent**

1. Cholesterol reagent
2. Cholesterol standard
3. Precipitating reagent
4. HDL-Cholesterol standard
Sample

9-12 hours fasting is recommended by national cholesterol education program, serum and plasma should be separated from cells within 3 hours of vein puncture. For plasma heparin or EDTA can be use as anticoagulants.

PROCEDURE

Step 1: HDL cholesterol separation

<table>
<thead>
<tr>
<th>Pipette in to tube marked</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum / Plasma</td>
<td>200 µl</td>
</tr>
<tr>
<td>Reagent -3</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

Mix well keeps at room temperature (15-30°C) for 10 minutes. Centrifuge for 15 minutes at 2000 rpm and separate clear supernatant. Use the supernatant for HDL Cholesterol estimation.

Step 2: HDL cholesterol estimation

<table>
<thead>
<tr>
<th>Pipette in to tube marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant from step 1</td>
<td>-</td>
<td>-</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Reagent -3</td>
<td>-</td>
<td>1000 µl</td>
<td>-</td>
</tr>
<tr>
<td>Reagent -1</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
</tbody>
</table>

Mix well keep incubate at 37°C for 10 minutes.

CALCULATION

HDL Cholesterol concentration (mg/dl) = Absorbance of Test/Absorbance of standard X 50X2

REFERENCE VALUE: 40-60 mg/dl.
CALCULATION OF LDL-CHOLESTEROL & VLDL CHOLESTEROL BY FRIEDWALD'S FORMULA (1972):

LDL-c and VLDL-c can be calculated from the following formula:

\[ \text{LDL-c} = \text{TC} - (\text{HDL-c} + \text{VLDL-c}) \]
\[ \text{VLDL-c} = \frac{\text{TG}}{5} \]

Reference Values

LDL-c = Up to 190 mg/dl (2.0 – 4.0 mmol/L)
VLDL-c = 14 -31.8 mg/dl (0.36 – 0.83 mmol/L)

ESTIMATION OF SERUM TOTAL BILIRUBIN

METHOD: (Enzymatic- colorimetric- dimethylsulphoxide (DMSO)-End Point- Mod. Jendrassik & Grof's Method.)

PRINCIPLE
The azobilirubin produced by the reaction between bilirubin and the diazonium salt of sulphanilic acid shows a maximum absorbance at 540 nm in an acid medium. The intensity of the purple colour produced is proportional to the quantity of total or direct bilirubin which has reacted. In the absence of an accelerator, only conjugated bilirubin reacts. In the presence of an accelerator, dimethylsulphoxide (DMSO) indirect bilirubin also participates in the reaction.
Direct/ conjugated bilirubin + Diazotized Sulphanilic acid → Purple colour azobilirubin complex

Indirect/ conjugated bilirubin + Diazotized Sulphanilic acid + DMSO → Purple colour azobilirubin complex

Reagent composition

1: Bilirubin direct
   - 100 ml
   - Sulphanilic acid 29 mMol/L
   - Hydrochloric acid 145 mMol/L

2: Bilirubin total DMSO
   - 100 ml
   - Sulphanilic acid 29 mMol/L
   - Hydrochloric acid 145 mMol/L
   - Dimethylsulphoxide (DMSO) 7 Mol/L

3: Sodium nitrite
   - 15 ml
   - Sodium nitrite 0.07 Mol/L

4: Standard (Artificial 9.2 mg/dL)
   - 50 ml

Sample: Serum free of haemolysis. Serum is stable 8 hrs. At 25°C and up to 3 days at 2-8°C.
PROCEDURE

<table>
<thead>
<tr>
<th></th>
<th>BLANK</th>
<th>DIRECT BILIRUBIN</th>
<th>TEST</th>
<th>TOTAL BILIRUBIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIRECT REAGENT-1</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TOTAL REAGENT-2</td>
<td>-</td>
<td>-</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>DISTILLED WATER</td>
<td>100 µl</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
</tr>
<tr>
<td>SODIUM NITRITE REAGENT-3</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
<td>100 µl</td>
</tr>
<tr>
<td>SAMPLE</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Read against reagent blank at wavelength 540 nm, Temperature 37°C.

Direct Bilirubin: Read immediately, No delay, after adding serum.

Total Bilirubin: Mix and after 10 minutes of incubation read the absorbance (A)

CALCULATION

Total bilirubin (mg/dl) - \[\frac{\text{OD of test} - \text{OD of blank}}{\text{OD of standard}}\] \times 9.2

Direct bilirubin (mg/dl) - \[\frac{\text{OD of test} - \text{OD of blank}}{\text{OD of standard}}\] \times 9.2

Direct bilirubin = Total bilirubin - Direct bilirubin

REFERENCE VALUES

Total bilirubin – 0.2 – 1.0 mg/dl

Direct bilirubin – 0.0 – 0.4 mg/dl
ESTIMATION OF SERUM GLUTAMATE PYRUVATE TRANSAMINASE

BY ENZYMATIC KIT METHOD: (MOD. IFCC METHOD)

PRINCIPLE:
Serum glutamate pyruvate transaminase (SGPT) in the sample originates, by means of the coupled reactions described below, a coloured complex that can be measured by spectrophotometry.

\[
\text{L-Alanine + } \alpha\text{-ketoglutarate } \xrightarrow{\text{SGPT}} \text{Pyruvate + L-Glutamate}
\]

\[
\text{Pyruvate + NADH + H}^+ \xrightarrow{\text{LDH}} \text{L-Lactate + NAD}^+
\]

\text{LDH – Lactate dehydrogenase}

Reagents Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer</td>
<td>100mMol/L</td>
</tr>
<tr>
<td>(\alpha)-ketoglutarate</td>
<td>15 mMol/L</td>
</tr>
<tr>
<td>L-aspartate</td>
<td>500 mMol/L</td>
</tr>
<tr>
<td>LDH</td>
<td>&gt;1200 U/L</td>
</tr>
<tr>
<td>NADH</td>
<td>0.24 mMol/L</td>
</tr>
<tr>
<td>L-alanine</td>
<td>500mMol/L</td>
</tr>
</tbody>
</table>

Contains stabilisers, preservatives and nonreactive fillers.

Working reagent is stable for 30 days, when stored at 2-8°C & protected from light.
Sample

Use fresh serum or heparinised (or EDTA Na+) plasma which shows no sign of haemolysis. SGPT activity reduces at a rate of 8%/hour at 2-8°C and 10% per hour at 30°C. Avoid fluoride plasma.

PROCEDURE

<table>
<thead>
<tr>
<th>Working Reagent</th>
<th>500 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

Mix well. Take one minute reading at 340 nm after a delay of 60 seconds at 37°C.

CALCULATIONS

\[ \text{Absorbance per minute} \times 1746 \]

System parameters

<table>
<thead>
<tr>
<th>Reaction</th>
<th>UV-Kinetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direction of Reaction</td>
<td>Inverse</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Wavelength</td>
<td>340 nm</td>
</tr>
<tr>
<td>Factor *</td>
<td>1746</td>
</tr>
<tr>
<td>Absorbance Range</td>
<td>0-2°A</td>
</tr>
<tr>
<td>Cuvette Path Length</td>
<td>1 cm</td>
</tr>
<tr>
<td>Delay Time</td>
<td>60 Seconds</td>
</tr>
</tbody>
</table>
Interval  
20 Seconds

Linearity  
300 U/L

Blank  
Distilled Water

REFERENCE VALUES:

Up to 44 IU/L

ESTIMATION OF ALKALINE PHOSPHATASE BY ENZYMATIC KIT METHOD: BASED ON DGKC AND SCE. (PNPP KINETIC METHOD)

PRINCIPLE:

In the presence of Mg++ and diethanolamine as phosphate acceptor, p-nitrophenylphosphate is transformed by alkaline phosphatases into inorganic phosphate and p-nitrophenol (yellow compound).

\[
\text{p- Nitrophenylphosphate} + \text{H}_2\text{O} \xrightarrow{\text{ALP}} \text{p- Nitrophenol} + \text{Pi}
\]

Reagents Composition

Nitrophenylphosphate  
10 mmol/L

Diethanolamine  
1 mol/L

\(\text{MgCl}\)  
0.5 mmol/L

Working reagent is stable for 30 days, when stored at 2-8°C & protected from light.
Sample

Use fresh serum or heparinised (or EDTA Na+) plasma which shows no sign of haemolysis. **Avoid fluoride plasma**

PROCEDURE

<table>
<thead>
<tr>
<th>Working Reagent</th>
<th>500 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Mix well. Take one minute reading at 405 nm after a delay of 60 seconds at 37°C.

CALCULATIONS

\[ = \text{Absorbance per minute} \times 2754 \]

SYSTEM PARAMETERS

- Reaction: Kinetic
- Direction of Reaction: Direct
- Temperature: 37°C
- Wavelength: 405nm (400-415 nm)
- Factor: 5454
- Absorbance Range: 0-2°A
- Cuvette Path Length: 1cm
- Delay Time: 60 seconds
Interval 20 seconds
Dynamic Range 10-1150 IU/L

REFERENCE VALUES:

Up to 260 IU/L

ESTIMATION OF γ (GAMMA) GLUTAMYL TRANSPEPTIDASE BY ENZYMATIC KIT METHOD: (Mod. IFCC method)

PRINCIPLE

GGT scatalyses the transfer of amino group between L-γ-glutamyl-carboxy-4-nitroanilide and glycylglycine to form L- γ-glutamylglycylglycine and 5-amino-2-nitrobenzoate. The rate of formation of 5-amino-2-nitrobenzoate is measured as an increase in absorbance which is proportional to the GGT activity in the sample.

L-γ-glutamyl-carboxy-4-nitroanilide $\xrightarrow{\text{GGT}}$ L- γ-glutamylglycylglycine
+ glycylglycine $\xrightarrow{}$ + 5-amino-2-nitrobenzoate
Reagents Composition

Glycylglycine 100mmol/L  
Tris preservative (pH 8.25) 95mmol/L  
L-γ-glutamyl-carboxy-4-nitroanilide 80mmol/L

Working reagent is stable for 15 days, when stored at 2-8°C & protected from light.

Sample

Use fresh serum or heparinised (or EDTA Na+) plasma which shows no sign of haemolysis. Avoid fluoride plasma.

PROCEDURE

<table>
<thead>
<tr>
<th>Working Reagent</th>
<th>1000 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Mix well. Take one minute reading at 405 nm after a delay of 60 seconds at 37°C.

CALCULATIONS

= Absorbance per minute x 1158
**System parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction</td>
<td>Kinetic</td>
</tr>
<tr>
<td>Wavelength</td>
<td>405nm</td>
</tr>
<tr>
<td>Zero Settings</td>
<td>distilled water</td>
</tr>
<tr>
<td>Incub. Temp.</td>
<td>370°C</td>
</tr>
<tr>
<td>Delay Time</td>
<td>30 second</td>
</tr>
<tr>
<td>Read Time</td>
<td>120 second</td>
</tr>
<tr>
<td>Interval</td>
<td>30</td>
</tr>
<tr>
<td>Sample Volume</td>
<td>100 μl</td>
</tr>
<tr>
<td>Reagent Volume</td>
<td>1000 μl</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
</tr>
<tr>
<td>Factor</td>
<td>1158</td>
</tr>
<tr>
<td>Linearity</td>
<td>700IU/L</td>
</tr>
</tbody>
</table>

**REFERENCE VALUES:**

Up to 40 IU/L
ENZYME IMMUNOASSAY FOR THE DETEMINATION OF PLASMA INSULIN

(LDN LABOR DIAGNOSTIKA NORD, ELISA Kit, GmbH & Co. KG)

PRINCIPLE OF THE ASSAY

The insulin ELISA kit is a solid phase enzyme – linked immunosorbent assay-linked immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the insulin molecule. An aliquot of patient sample containing endogenous insulin is incubated in the coated well with enzyme conjugate is washed off. During the second incubation step streptavidin peroxidise enzyme complex binds to the biotin –anti-insulin antibody. The amount of bound HRP complex is proportional to the concentration of insulin in the sample. Having added the substrate solution, the intensity of colour developed is proportional to the concentration of insulin in the patient sample.

Preparation of Reagents

Allow all reagents and required number of strips to reach room temperature prior to use.
Wash Solution

Dilute 30 ml of concentrated wash solution with 1170 ml deionised water to a final volume of 1200ml. The diluted wash solution is stable for 2 weeks at room temperature.

SPECIMEN

Serum or plasma (only heparin – or citrate plasma) can be used in this assay. Do not use haemolytic, icteric or lipemic specimen.

Specimen collection

Serum

Collect blood by venipuncture (e.g. Sarsted Monovette # 02.1388.001), allow to clot, and separate serum by centrifugation at room temperature.

Plasma

Whole blood should be collected in to centrifuge tubes containing anti coagulant and centrifuged immediately after collection.

Specimen storage

Specimen should be capped and may be stored for up to 5 days at room temp. 2-8°C prior to assaying. Specimen held for a longer time should be frozen only once -20°C prior to assay.
Specimen dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted 10-fold or 100-fold with zero standard and reassayed as described in assay procedure. For the calculation of the concentrations this dilution factor has to be taken into account.

Example

A) Dilution 1:10: 10 µl serum + 90 µl standard Zero standard (mix thoroughly)

b) Dilution 1:100: 10 µl dilution a) 1:10 + 90 µl zero (mix thoroughly).

ASSAY PROCEDURE

1. Secure the desired number of microtiter wells in the holder.

2. Dispense 25µl of each standard controls and samples with new disposable tips into appropriate wells.

3. Dispense 25 µl Enzyme conjugate into each well.

4. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.

5. Incubate for 30 minutes at room temperature without covering the plate.

6. Briskly shake out the contents of the wells.
7. Rinse the wells 3 times with diluted wash solution (400 µl per well). Strike the wells sharply on absorbent paper to remove residual droplets.

8. Add 50 µl of enzyme complex to each well.

9. Incubate for 30 minutes at room temperature.

10. Briskly shake out the contents of the wells. Rinse the wells 3 times with diluted wash solution (400 µl per well). Strike the wells sharply on absorbent paper to remove residual droplets.

11. Add 50 µl of substrate solution to each well.

12. Incubate for 15 minutes at room temperature.

13. Stop the enzymatic reaction by adding 50 µl of stop solution to each well.

14. Read the OD at 450± nm with a microtiter plate reader within 10 min after adding the stop solution.

**Calculation of Results**

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (y) axis and concentration on the horizontal (x) axis.
3. using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted. For the calculation of the concentrations this dilution factor has to be taken into account. Below is listed a typical example of a standard curve with the Insulin ELISA.

<table>
<thead>
<tr>
<th>STANDARD</th>
<th>Optical Units (450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 0 (0 µIU/ml)</td>
<td>0.03</td>
</tr>
<tr>
<td>Standard 1 (6.25 µIU/ml)</td>
<td>0.07</td>
</tr>
<tr>
<td>Standard 2 (12.5 µIU/ml)</td>
<td>0.14</td>
</tr>
<tr>
<td>Standard 3 (25 µIU/ml)</td>
<td>0.35</td>
</tr>
<tr>
<td>Standard 4 (50 µIU/ml)</td>
<td>0.88</td>
</tr>
<tr>
<td>Standard 5 (100 µIU/ml)</td>
<td>2.05</td>
</tr>
</tbody>
</table>
Expected values

It is strongly recommended that each laboratory should determine its own normal and abnormal values. In a study conducted with apparently normal healthy adults, using the insulin ELISA the following values are observed:

2 to 25 µIU/ml.

Insulin Resistance

Insulin resistance values were derived using the homeostasis model assessment (HOMA) method, employing the equation below.

\[ IR = \frac{(\text{Fasting plasma insulin micro units/L}) \times (\text{Plasma fasting glucose mmol/L})}{22.5} \]

Or

\[ IR = \frac{(\text{Fasting plasma insulin micro units/L}) \times (\text{Plasma fasting glucose mg/dl})}{405} \]
ESTIMATION OF SERUM HIGH SENSITIVE C-REACTIVE PROTEIN
BY LATTEX-IMMUNOTURBIDIMETRIC HIGH SENSITIVITY
METHOD: (POINTE SCIENTIFIC, INC. 5449 RESEARCH DRIVE,
CANTON, MI 48188)

PRINCIPLE OF THE ASSAY

Serum C-reactive protein (hs-CRP) cases agglutination of the latex
particles coated with anti-human C-reactive protein. The agglutination of the
latex particles is proportional to the CRP concentration and can be measured
by turbidimetry.

Reagents

A. Glycine buffer (0.1 mol/L), Sodium Azide (0.95gm/L) pH 8.6

B. Suspension of latex particles coated with anti-human C-reactive protein
antibodies, Sodium Azide (0.95gm/L)

C. hs-CRP Standard

Reagent Storage and Stability

1. All reagents should be stored at 2-8°C and protected from light.

2. Unopened reagents can be used until the expiration date on the package
   and bottle labels.

3. Once the reagent vial has been opened, store tightly capped at 2-8°C
   and use within 1 month.
SPECIMEN

Serum or plasma (only heparin) can be used in this assay. Do not use haemolytic, icteric or lipemic specimen.

Specimen collection

Serum

Collect blood by venipuncture (e.g. Sarsted Monovette # 02.1388.001), allow to clot, and separate serum by centrifugation at room temperature.

Plasma

Whole blood should be collected in to centrifuge tubes containing anti coagulant and centrifuged immediately after collection.

Specimen storage

Specimen should be capped and may be stored for up to 7 days at room temp. 2-8°C prior to assaying. Specimen held for a longer time should be frozen only once -20°C prior to assay.

Specimen dilution

Calibration curve: Prepare dilution of the hs CRP Standard using 9g/L saline as diluents. Multiply the concentration of the hs CRP Standard by the corresponding factor indicated below to obtain the hs CRP concentration of the dilution.
<table>
<thead>
<tr>
<th>Dilution</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard (µl)</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>Saline (µl)</td>
<td>70</td>
<td>60</td>
<td>40</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Factor</td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**ASSAY PROCEDURE**

<table>
<thead>
<tr>
<th>Sample standard/ water(blank)</th>
<th>20 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent (9ml reagent A + 1ml reagent B)</td>
<td>1.5 ml</td>
</tr>
</tbody>
</table>

Mix and immediately insert the cuvette into the instrument. Start stopwatch. Record the absorbance at 540 nm and after 10 seconds (A1) and after 5 seconds (A2)

**Calculation**

Calibration curve: Calculate the absorbance difference ($A_{\text{standard}} - A_{\text{blank}}$) of each point of the calibration curve and plot the values found against the hs-CRP concentration. hs-CRP concentration in the sample is calculated by interpolation of its absorbance ($A_{\text{standard}} - A_{\text{blank}}$) on the calibration curve.
Expected values

It is strongly recommended that each laboratory should determine its own normal and abnormal values. In a study conducted with apparently normal healthy adults, using the Serum hs-CRP latex-immunoturbidimetric the following values are observed: up to 6.5 mg/L.

Statistical analysis

Statistical Package for the Social science 21 (SPSS 21, Chicago, Illinois, USA) was used for all statistical analysis. All parameters were given as mean±standard deviation (S.D.). The comparisons between two groups were analyzed by Student’s t-test. Correlations between variables were analyzed by Pearson’s correlation coefficient test $P < 0.05$ and $P < 0.01$ was considered significant in nonalcoholic fatty liver obese type II diabetic subjects.