MATERIAL AND METHODS

3.1 Source of data

The present case control study was done during the period from 1st January 2010 to 31st January 2011. All the participants were recruited from three hospitals (civil hospitals of Amritsar, Jalandhar and Kapurthala). The study was done on a total of two hundred subjects including 100 diabetic (20 type 1 and 80 type 2) and 100 healthy non diabetic subjects. Written consent was obtained from each subject after explaining the objectives as well as methodology of study. Copy of Consent form is given in Annexure I.

3.2 Specimen collection and preservation

Participants were required to fast at least twelve hours before the blood sample was taken. 6 ml of whole blood was collected from each subject and samples were further divided into two aliquot, one of 2 ml in EDTA and other of 4 ml in plain dry vacutainer. Serum was separated from the cells within 30 minutes and was further used for estimation of lipid profile, renal profile, thyroid test and liver function test. The EDTA sample was used for the estimation of Hb, TLC, FPG and HbA1C.

3.3 Inclusion criteria

A comprehensive survey of literature was done from various research journals to design the performa. A copy of performa has been given in Annexure II. In the construction of performa atmost care was taken to make it broad based so that all aspects desired to be studied could be incorporated in its body. Information about age, disease history, age at diagnosis of diabetes was collected for each subject. Inclusion criterion for diabetic subjects was levels of FPG more than 110 mg % at more than two occasions. All the diagnosis were made by physicians. Healthy subjects were selected by random selective sampling from different areas of Amritsar, Jalandhar and Kapurthala.
3.4 Exclusion criteria

a. Patient suffering from any type of rheumatoid arthritis, tuberculosis, collagen diseases, liver diseases, renal diseases, cardiac failure and gout were not included in the present study.

b. Patient suffering from any type of thyroid diseases such as hypothyroidism and hyperthyroidism were also not included in the present study.

c. Very ill patients with complication of diabetes mellitus.

3.5 Investigations done

3.5.1 Routine

The following routine investigation were done

3.5.1.1 Hemoglobin (Hb) %

The hemoglobin was determined by Cyanmethemoglobin method following the method of Dacie & Lewis (1975).

3.5.1.1.1 Principle: Hemoglobin is converted into Cyanmethemoglobin by the addition of KCN and ferricyanide. The colour of Cyanmethemoglobin is read in a photoelectric colorimeter at 540 nm against a standard solution. Science cyanide has the maximum affinity for hemoglobin, this method estimates, the total hemoglobin.

3.5.1.1.2 Reagents

**Drabkin’s solution:** 0.05 g of KCN, 0.2 g of potassium ferrocyanide and 1 g of sodium bicarbonate was dissolved in 1 lt. of distilled water.

**Standard solution:** Standard Cyanmethemoglobin ampoule.

3.5.1.1.3 Procedure: 20 µl of blood was transferred with the help of hemoglobin pipette into a test tube containing 5 ml of Drabkin’s solution. After adjusting the photoelectric colorimeter at 540 nm with a blank (Drabkin’s diluents) the O.D. of sample was read.
The standard solution in the ampoule contain 14.8 g of hemoglobin/100 ml. The corresponding blood hemoglobin in g/100 ml was obtained by multiplying the concentration of the ampoule by the dilution factor.

3.5.1.2 Total leucocyte count (TLC)

The total leucocyte count was determined by haemocytometry.

3.5.1.2.1 Principle The glacial acetic acid lyses the red cells while the gentian violet slightly stains the nuclei of the leucocyte. The blood specimen is diluted to 1:20 in a WBC pipette with the diluting fluid and the cells are counted under low power microscope by using a counter chamber. The, number of cells in undiluted blood are reported as the number of white cells/cu.mm of whole blood.

The additional requirements are the following:

1. WBC pipette
2. WBC diluting fluid.

This contains 1% acetic acid solution tinged with Gentian violet stain. Acetic acid facilitates haemolysis of RBC and Gentian violet stains the nuclei of RBC.

3.5.1.2.2 Procedure

WBC diluting fluid was taken in a watch glass. Capillary blood was obtained by pricking the finger with the lancet. Blood was drawn upto 0.5 mark of the WBC pipette and WBC diluting fluid was drawn upto 11 mark. The fluid and blood were mixed well and the first few drops of blood were discarded by holding the pipette vertically. The counting chamber was charged with a drop of blood mixed with diluting fluid. The chamber was let undisturbed for few minutes. The four corners of the chamber was visualized under a low power (10x) objective and the cells were counted in all the four marked corner squares.

3.5.1.2.3 Calculation

\[
\text{Total WBC / cu mm} = \frac{\text{Number of cells counted} \times \text{dilution factor}}{\text{Area counted} \times \text{depth of fluid}}
\]
where Dilution factor = 1:20
Area counted = 1/5 sq.mm
Depth of fluid = 1/10 mm
Number of white cells counted = N

3.5.2 Liver function tests

The following liver related investigation was done

3.5.2.1 Serum glutamate oxaloacetate transaminase (SGOT): The estimation of SGOT was done by IFCC methods of Bergmeyer et al., (a) (1986).

3.5.2.1.1 Principle

α-ketoglutarate reacts with L-aspartate in presence of GOT (AST) to form oxaloacetate and L-glutamate. The increase in oxaloacetate is determined in an indicator reaction catalyzed by malate dehydrogenase. The conversion of NADH to NAD, at 340 nm, is proportional to the activity of GOT (AST) in serum/plasma and is determined kinetically as rate of decrease in absorbance.

\[
\text{L-aspartate + } \alpha\text{-ketoglutarate } \xrightarrow{\text{GOT (AST)}} \text{oxaloacetate + L-glutamate}
\]

\[
\text{Oxaloacetate + NADH + H}^+ \xrightarrow{\text{Malate dehydrogenase}} \text{L-malate + NAD}^+
\]

Abbreviations

AST = Aspartate transaminase
GOT = Glutamate oxaloacetate transaminase

3.5.2.1.2 Preparation of working solution

Prepared working solution by mixing *Reagent R₁* and *R₂* in the ratio 4 : 1 as per requirement.
3.5.2.1.3 Components and concentration of working solutions

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer, pH 7.8</td>
<td>80 mmol/l</td>
</tr>
<tr>
<td>L-aspartate</td>
<td>240 mmol/l</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>≥ 3000 IU/l</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>≥ 400 IU/l</td>
</tr>
<tr>
<td>NADH</td>
<td>0.23 mmol/l</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>10 mmol/l</td>
</tr>
</tbody>
</table>

3.5.2.1.4 Parameters

- Reaction type: UV - Kinetic
- Reaction direction: Decreasing
- Wavelength: 340 nm
- Flowcell temp: 37°C
- Zero setting with: Distilled water
- Delay time: 60 seconds
- No. of readings: 4
- Interval: 30 seconds
- Blank absorbance limit: ≥ 0.900 Abs.
- Sample volume: 0.05 ml (50 µl)
- Working solution Volume (4 R₁:R₂): 1.0ml (1000 µl)
- Factor: 3339
- Linearity: 800 IU/l
3.5.2.1.5 Manual assay procedure

Pre warmed at 37°C the required amount of working solution before use. Performed the assay as given below

1 ml procedure

Serum……………………………………0.05 ml.

Working solution…………………1.0 ml (800 µl R₁ + 200 µl R₂)

Mixed thoroughly and transferred the assay mixture immediately to the thermostated cuvette and start the stop watch simultaneously. Recorded the first reading at 60th second and subsequently three more readings with 30 seconds interval at 340 nm.

3.5.2.1.6 Calculation

Calculated the change in absorbance per minute.

\[(\Delta \text{Abs.}/30 \text{ seconds} \times 2)\]

Activity of GOT (AST) in IU/l = \(\Delta \text{Abs.}/\text{min.} \times 3339\)

3.5.2.2 Serum glutamate pyruvate transaminase (SGPT)

The estimation of SGPT was determined by IFCC method of Bergmeyer et al., (b) (1986)

3.5.2.2.1 Principle

\[
\begin{align*}
\text{L-Alanine} + \alpha - \text{Ketoglutarate} & \xrightarrow{\text{GPT}} \text{Pyruvate} + \text{L-Glutamate} \\
\text{Pyruvate} + \text{NADH} + \text{H}^+ & \rightarrow \text{L – Lactate} + \text{NAD}^+
\end{align*}
\]

3.5.2.2.2 Components of reagent

The following components are present

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Buffer, pH 7.7</td>
<td>80 mmol/l</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>200 mmol/l</td>
</tr>
<tr>
<td>NADH</td>
<td>0.15 mmol/l</td>
</tr>
</tbody>
</table>
Material and Methods

- LDH…………………………………… 2000 IU/l
- A-ketoglutarate……………………. 12 mmol/l
- Stabilizers, inactive ingredients and surface active agents.

3.5.2.2.3 Preparation, storage and stability

Reagent R1 and R2 were ready to use liquid reagents. Mixed the reagents R1 and R2 in ratio of 4:1 respectively to prepare the desired volume of working reagents.

3.5.2.2.4 Text parameters

- Reaction Type………………………..Kinetic (↓)
- Wavelength……………………………340 nm
- Flow cell temp………………………...37°C
- Blank abs. limit……………………..>0.900
- Blank setting………………………..D/W
- Linearity…………………………….600 IU/l
- Reagent vol……………………….1.0 ml
- Sample vol…………………………50 µl
- Delay time…………………………..60 sec
- Interval time………………………..30 sec.
- No. of readings……………………..4
- Factor………………………………3339

3.5.2.2.5 Manual assay procedure

Prewarmed the working reagent at 37°C before use. Performed the assay as given below

- Reagent……………..1.0 ml.
- Serum ………………0.05 ml (50 µl).
Material and Methods

Started stop watch:

Mixed and transferred assay mixture to themostated cuvette.

Recorded absorbance at 60, 90, 120 & 150\textsuperscript{th} sec. (30 sec. interval).

**3.5.2.2.6 Calculation**

Calculated the change in absorbance per minute.

\[(\Delta \text{Abs} / 30 \text{ seconds} \times 2)\]

\[\text{GPT (IU/L)} = \Delta \text{Abs} / \text{min} \times 3339\]

**3.5.3 Lipid profile**

The following investigation were done under lipid profile

**3.5.3.1 Cholesterol**

The estimation of serum cholesterol was done by end point method of Allain \textit{et al.} (1974).

**3.5.3.1.1 Principle:** Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. In the second reaction cholesterol oxidase converts cholesterol to cholest-4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidatively couple with 4-aminoantipyrine and phenol to produce red quinoneimine dye which was absorbance (maximum at 510nm, 505-530 nm). The intensity of the red colour is proportional to the amount of total cholesterol in the specimen.

\[
\begin{align*}
\text{Cholesterol Esters} & \xrightarrow{\text{CHE}^*} \text{Cholesterol + Fatty Acids} \\
\text{Cholesterol + O}_2 & \xrightarrow{\text{CHO}^*} \text{H}_2\text{O}_2 + \text{Cholest-4-en-3-one} \\
2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine + Phenol} & \xrightarrow{\text{POD}^*} \text{Red Quinoneimine Dye+ H}_2\text{O} 
\end{align*}
\]

*Abbreviations
Material and Methods

CHE = Cholesterol Esterase
CHO = Cholesterol Oxidase
POD = Peroxidase

3.5.3.1.2 Components and concentration of working solutions

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer; pH 7.5</td>
<td>100 mmol/l</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>≥ 100 IU/l</td>
</tr>
<tr>
<td>Cholesterol esterase</td>
<td>≥ 150 IU/l</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥ 500 IU/l</td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>0.5 mmol/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>≥ 10 mmol/l</td>
</tr>
</tbody>
</table>

3.5.3.1.3 Procedure

- Reaction type..........................End Point
- Reaction time.......................... 5 mins. at 37°C
- Wavelength............................510 nm. (505-530 nm.)
- Zero setting with......................Working Solution
- Blank absorbance limit...............< 0.300 Abs.
- Sample volume..........................1.0 ml
- Standard concentration...............200 mg/dl
- Linearity................................1000 mg/dl

3.5.3.1.4 Manual assay procedure

Prewarmed at room temperature (25-30°C) the required amount of working reagent before use. Performed the assay as given below

63
Material and Methods

1.0 ml procedure

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working solution</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

3.5.3.1.5 Incubation

Incubate the assay mixture for 5 minutes at 37°C. After incubation the absorbance of assay mixture was measured against blank at 510 nm.

3.5.3.1.6 Calculation

Total Cholesterol in mg/dl = \[ \frac{\text{Absorbance of Sample} \times 200}{\text{Absorbance of Standard}} \]

3.5.3.2 Triglyceride

The estimation of triglycerides was done by end point method of McGowan et al., (1983).

3.5.3.2.1 Principle: Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol - 3 - phosphate which is oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red coloured compound.

\[ \text{Triglycerides} \xrightarrow{\text{LPL}^*} \text{Fatty acids + Glycerol} \]

\[ \text{Glycerol + ATP} \xrightarrow{\text{GK}^*} \text{Glycerol-3-phosphate + ADP} \]

\[ \text{Glycerol-3-phosphate + O}_2 \xrightarrow{\text{GPO}^*} \text{Dihydroxyacetone phosphate + H}_2\text{O}_2 \]

\[ \text{H}_2\text{O}_2 + \text{Phenol chromogens} \xrightarrow{\text{POD}^*} \text{Red colour compound} \]

*Abbreviations

LPL = Lipoprotein Lipase  \hspace{1cm} GPO = Glycerol phosphate Oxidase
Material and Methods

GK = Glycerol Kinase  POD = Peroxidase

3.5.3.2.2 Preparation of working solution

Reconstituted reagents as per instruction on individual bottle label to prepare working reagent.

3.5.3.2.3 Components and concentration of working solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer, pH 6.8</td>
<td>50 mmol/l</td>
</tr>
<tr>
<td>Lipase</td>
<td>≥ 2000 IU/l</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>≥ 300 IU/l</td>
</tr>
<tr>
<td>Glycerol phosphate oxidase</td>
<td>≥ 1000 IU/l</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥ 500 IU/l</td>
</tr>
<tr>
<td>ATP</td>
<td>1 mmol/l</td>
</tr>
<tr>
<td>Chromogens</td>
<td>2 mmol/l</td>
</tr>
</tbody>
</table>

3.5.3.2.4 Procedure

- Reaction type…………………………..End Point
- Reaction time…………………………..10 mins.at 37°C
- Wavelength……………………………..510 nm. (500530 nm.)
- Zero setting with…………………….. Reagent Blank
- Blank absorbance limit………………..< 0.20 Abs.
- Sample volume………………………..0.01 ml (10 µl)
- Reagent volume……………………….1.0ml
- Standard concentration………………..200 mg/dl
- Linearity……………………………..800 mg/dl

3.5.3.2.5 Manual assay procedure

Pre warmed at room temperature (25-30°C) the required amount of working reagent before use. Performed the assay as given below:
Material and Methods

1.0 ml procedure

<table>
<thead>
<tr>
<th></th>
<th>Serum/ Plasma</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working solution</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

3.5.3.2.6 Incubation

Incubated the assay mixture for 10 minutes at 37°C. The absorbance was measured against blank at 510 nm. (500-530nm).

3.5.3.2.7 Calculation

\[
\text{Triglycerides in mg/dl} = \frac{\text{Absorbance of Sample} \times 200}{\text{Absorbance of Standard}}
\]

3.5.3.3 HDL-cholesterol

The estimation of serum HDL was done by precipitation method of Lopez-Virella et al. (1977).

3.5.3.3.1 Principle: phosphotungstate/Mg\(^{2+}\) precipitates chylomicrons, LDL and VLDL fractions. High Density Lipoprotein (HDL) fraction remains unaffected in supernatant. Cholesterol content of HDL fraction is assayed using Autozyme cholesterol.

3.5.3.3.2 Components and concentration of precipitating reagent

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphotungstic acid</td>
<td>2.4 mmol/l</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>40 mmol/l</td>
</tr>
</tbody>
</table>

[Diagram: Phosphotungstate + Mg\(^{2+}\) → HDL Fraction+ (LDL+VLDL+Chylomicrons) (Supernatant) (precipitate)
3.5.3.3.3 Procedure

HDL separation

Pre warmed at room temperature (25 - 30°C) the required amount of precipitating reagent and AutoZyme cholesterol working solution before use. Performed the assay as the given below

Pipetted as follows

<table>
<thead>
<tr>
<th>Serum/plasma</th>
<th>0.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-Precipitating reagent</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Mixed thoroughly and centrifuged at 4000 r.p.m. for 10 minutes in a common laboratory centrifuge (1800 x g) to obtain a clear supernatant.

3.5.3.3.4 HDL-cholesterol determination

- Reaction type…………………………………End Point
- Reaction time…………………………………10 mins.at 37°C
- Wavelength…………………………………510 nm. (505-530 nm.)
- Zero setting with……………………………Reagent Blank
- Blank absorbance limit………………………< 0.100 Abs.
- Sample volume……………………………0.05 ml (50 µl)
- Reagent volume……………………………1.0 ml
- Standard concentration……………………50 mg/dl
- Linearity…………………………………400 mg/dl

3.5.3.3.5 Manual assay procedure

Assayed the supernatant for HDL-cholesterol after centrifugation using working solution of AutoZyme cholesterol reagent.
1.0 ml procedure

<table>
<thead>
<tr>
<th></th>
<th>Supernatant</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 ml</td>
<td>0.05 ml</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Autozyme Cholesterol</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Working Solution</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.5.3.6 Incubation

Incubated the assay mixture for 10 minutes at 37°C. After completion of the incubation, the absorbance of assay mixture was measured against blank at 510 nm.

3.5.3.7 Calculation

\[
\text{HDL-Cholesterol in mg/dl} = \frac{\text{Absorbance of Sample} \times 200}{\text{Absorbance of Standard}}
\]

Factor of 200 was used instead of 50 for calculation due to serum dilution during precipitating step.

3.5.3.4 LDL-cholesterol

The estimation of LDL-cholesterol was determined by using formula of Friedwald et al. (1972) as shown below:

\[
\text{LDL-cholesterol} = \frac{\text{Total cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL-cholesterol}}{5}
\]

3.5.3.5 VLDL-cholesterol

The estimation of VLDL-cholesterol was done by following formula as shown below:

\[
\text{VLDL} = \frac{\text{Triglycerides}}{5}
\]

3.5.4 Renal function tests

The following renal related tests were done
3.5.4.1 Blood urea

The estimation of blood urea was done by kinetic method of Fawcett and Scott, (1960).

3.5.4.1.1 Principle

The enzymatic reactions involved in the BUN assay are as follow

\[
\text{Urea} + \text{H}_2\text{O} \xrightarrow{\text{Urease}} 2\text{NH}_3 + \text{CO}_2
\]

\[
\text{NH}_3 + \alpha\text{-ketoglutarate} + \text{NADH} \xrightarrow{\text{GLDH}} \text{Glutamate} + \text{NAD}
\]

GLDH: Glutamate dehydrogenase

Urea is hydrolyzed to ammonia and carbon dioxide by urease. Ammonia produced reacts with \(\alpha\)-ketoglutarate to form glutamate in presence of glutamate dehydrogenase. NADH is oxidized to NAD\(^+\) in this reaction, which is measured as decrease in absorbance at 340 nm. The rate of decrease in absorbance at 340 nm is directly proportional to BUN concentration in the specimen. The BUN concentration in the specimen is determined by comparing the sample reaction rate to that obtained with a BUN standard.

3.5.4.1.2 Components and concentration of working solutions

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Buffer, pH 7.7</td>
<td>50 mmol/l</td>
</tr>
<tr>
<td>(\alpha)-ketoglutarate</td>
<td>10 mmol/l</td>
</tr>
<tr>
<td>NADH</td>
<td>25 mmol/l</td>
</tr>
<tr>
<td>Urease</td>
<td>10 KU/l</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>900 U/l</td>
</tr>
<tr>
<td>Stabilizers and inactive ingredients</td>
<td></td>
</tr>
</tbody>
</table>
3.5.4.1.3 Procedure

- Reaction type……………………………….UV - Kinetic
- Reaction time……………………………….Down
- Wavelength……………………………….340
- Flowcell temperature……………………..30°C
- Zero setting with…………………………. Distilled water
- Delay time…………………………………30 seconds
- No. of readings…………………………….2
- Interval……………………………………30 seconds
- Blank absorbance limit……………………≥ 1.000 Abs.
- Sample volume……………………………0.01 ml (10 µl)
- Reagent Volume…………………………..1.0ml
- Factor……………………………………….20 ÷ (∆ Abs. of Std)
- Linearity…………………………………….250 mg/dl BUN

3.5.4.1.4 Manual assay procedure

Pre warmed at 30°C the required amount of working solution before use.

Performed the assay as given below:

1.0 ml procedure

<table>
<thead>
<tr>
<th>Standard/Specimen</th>
<th>0.01 ml (10µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working solution</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

First carried out the assay of standard, mixed and started stopwatch simultaneously. The absorbance of assay was recorded at exactly 30 seconds after standard addition and then again at 60 seconds. Subsequently, carried out the assay of the specimen, following exactly the same procedure mentioned above.
Material and Methods

3.5.4.1.5 Calculation

Calculated the change in absorbance (Δ Abs.) of Standard and Specimen (s).

\[
\begin{align*}
\text{Factor} &= \frac{\text{Concentration of Standard}}{\Delta \text{Abs. of Standard}} \\
&= \frac{20}{\Delta \text{Abs. of Standard}}
\end{align*}
\]

BUN mg/dl = ΔAbs. of Specimen × Factor

Blood urea = BUN × 2.14

3.5.4.2 Serum creatinine

The estimation of serum creatinine was done by picrate method of Owen et al.(1954).

3.5.4.2.1 Principle: Creatinine in alkaline medium reacts with picrate to produce orange colour. This colour absorbs light at 492 nm (490 - 510 nm). The rate of increase in absorbance is directly proportional to the concentration of creatinine in specimen.

\[
\text{Alkaline medium} \quad \text{Creatinine} + \text{Picrate} \rightarrow \text{Orange colour}
\]

3.5.4.2.2 Preparation of working solution

Prepared working solution by mixing equal volume of picrate reagent and diluent reagent.

3.5.4.2.3 Components and concentration of working solutions

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Picrate</td>
<td>7.7 mmol/l</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>500 mmol/l</td>
</tr>
</tbody>
</table>

3.5.4.2.4 Procedure

- Reaction type...........................................Initial rate
- Reaction time..........................................Up
Material and Methods

- Wavelength……………………………………492 nm. (490-510 nm.)
- Flowcell temperature………………………… 37°C
- Zero setting with…………………………… Distilled water
- Delay time……………………………………30 seconds
- No. of readings………………………………………2
- Interval…………………………………………60 seconds
- Sample volume……………………………………0.05 ml (50 µl)
- Reagent Volume……………………………………1.0ml
- Standard concentration…………………………2 mg/dl
- Factor………………………………………2 ÷ Δ Abs. of Standard
- Linearity…………………………………………30 mg/dl

3.5.4.2.5 Manual assay procedure

Pre warmed the required amount of working solution to 37°C before use. Performed the assay as given below

1.0 ml procedure

<table>
<thead>
<tr>
<th>Standard / Sample</th>
<th>0.05 ml (50 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Solution</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Mixed and started stopwatch simultaneously. The absorbance of assay mixture was recorded at exactly 30 seconds after standard / specimen addition and then again at 90 seconds.

3.5.4.2.6 Calculation:

Calculated the average change in absorbance per minute (Δ Abs.) of standard & specimen (s).

Δ Abs. = Abs. at 90 sec. — Abs. at 30 sec.
Material and Methods

\[ \text{Serum Creatinine (mg/dl)} = \frac{\Delta \text{Abs. of Specimen} \times 2}{\Delta \text{Abs. of Standard}} \]

3.5.5 Diabetes related

The following investigations were included under diabetes related tests

3.5.5.1 Fasting plasma glucose (FPG) level

The estimation of fasting plasma glucose (FPG) level was done by GOD-POD method of Trinder (1969).

3.5.5.1.1 Principle: Glucose oxidase (GOD) converts glucose to gluconic acid. Hydrogen peroxide formed in this reaction, in presence of peroxidase (POD) oxidatively couples with 4-aminoantipyrine and phenol to produce red quinoneimine dye. This dye has absorbance maximum at 505 nm (500-550 nm). The intensity of the colour complex is directly proportional to the concentration of glucose in sample.

\[
\begin{align*}
\beta - \text{D Glucose} + O_2 + H_2O & \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2 \\
H_2O_2 + 4\text{-aminoantipyrine} + \text{phenol} & \xrightarrow{\text{POD}} \text{Red Dye} + H_2O
\end{align*}
\]

3.5.5.1.2 Components and concentration of working solutions

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Buffer, pH 7.0</td>
<td>170 mmol/l</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>15000 IU/l</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>1500 IU/l</td>
</tr>
<tr>
<td>4 – amino antipyrine</td>
<td>0.28 mmol/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>16 mmol/l</td>
</tr>
<tr>
<td>Stabilizers and inactive ingredients</td>
<td></td>
</tr>
</tbody>
</table>
3.5.5.1.3 Procedure

- Reaction type………………………………………………..End Point
- Reaction time………………………………………………7 mins. at 37°C
- Wavelength………………………………………………505 nm. (500 - 530 nm.)
- Zero setting with……………………………..Reagent Blank
- Blank absorbance limit…………………………..< 0.300 Abs.
- Sample volume…………………………………………0.01 ml (10 µl)
- Reagent Volume………………………………………1.0 ml
- Standard concentration…………………………100 mg/dl
- Linearity………………………………………………500 mg/dl

3.5.5.1.4 Manual assay procedure

Pre warmed at room temperature (25-30°C) the required amount of reagent before use. Performed the assay as given below

1.0 ml procedure

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 ml</td>
<td>0.01 ml</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

3.5.5.1.5 Incubation

Incubated the assay mixture for 7 minutes at 37°C. After completion of incubation period the absorbance was measured against blank at 505 nm.
3.5.5.1.6 Calculation

With standard

\[
\frac{\text{Absorbance of Sample} \times 100}{\text{Absorbance of Standard}}
\]

3.5.5.2 Glycosylated hemoglobin (HbA1C)

The estimation of glycosylated hemoglobin was done by Ion Exchange Resin method by Trivelli et al. (1971).

3.5.5.2.1 Principal: - Whole blood is mixed with the lysing reagent to prepare a haemolysate this is then mixed with a weakly binding cation-exchange resin. Non-glycosylated hemoglobin binds to the resin leaving GHb (Glycosylated Hemoglobin) free in the supernatant. The GHb percentage is determined by measuring the absorbance of the GHb faction to that of the total hemoglobin.

3.5.5.2.2 Reagent components

R1: Lysing reagent (Triton x 100)

R2: Ion Exchange Resin (CM Sephadex, Sodium Hyroxide)

3.5.5.2.3 Specimen collection & preservation

Whole blood was collected in a clean and dry container with EDTA.

3.5.5.2.4 Procedure

- Reaction type……………………………………………………End Point
- Wavelength……………………………………………..415 nm.
- Assay temperature…………………………………….. 30\(^{0}\)C (±1\(^{0}\)C)
- Zero setting with……………………………………..Deionized water
- Sample volume………………………………………..0.5 ml (50 µl)
- R1 volume…………………………………………….0.25 ml (250 µl)
- R2 volume…………………………………………….3.00 ml (3000 µl)
- Linearity………………………………………………20 \(^{0}\)
3.5.5.2.5 Manual assay procedure

Performed the assay as given below:

**Step I – Haemolysate preparation**

<table>
<thead>
<tr>
<th>Haemolysate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>0.25 ml (250 µl)</td>
</tr>
<tr>
<td>Sample / Control</td>
<td>0.05 ml (50 µl)</td>
</tr>
</tbody>
</table>

Mixed well and allowed it to stand at R.T (25-30°C) for 5 minutes.

**Step II- GHb separation and assay**

1. Brunged the resin tube (R2) to assay temperature (30°C ± 1°C).
2. Added 0.1 ml (100 µl) of haemolysate from Step I to it.
3. Positioned a resin separator in the tube such that the rubber sleeve was approximately 3 cms above the resin level.
4. Mixed the contents on vortex mixer continuously for 5 minutes.
5. Allowed the resin to settle at assay temperature (30°C ± 1°C) for 50 minutes.
6. Pushed down the resin separator in the tube until the resin was firmly packed.
7. Poured the supernatant directly into a cuvette and the absorbance was measured at 415 nm against deionized water.

**Step III – Total Hemoglobin (THb) assay**

<table>
<thead>
<tr>
<th>Total Hemoglobin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>5.00 ml</td>
</tr>
<tr>
<td>Haemolysate</td>
<td>0.02 ml (20 µl)</td>
</tr>
</tbody>
</table>

Mixed and absorbance was read at 415 nm. against deionized water.
Material and Methods

3.5.5.2.6 Calculation:

\[
\text{GHb \%} = \frac{\text{Abs. of GHb}}{\text{Abs. of THb}} \times 10 \times \text{Temperature factor (Tf)}
\]

For assay at 30°C Tf = 0.9

3.5.6 Thyroid related

The following investigations were included under thyroid related tests

3.5.6.1 Total triiodothyronine (T₃)

The estimation of T₃ was done by electrochemiluminance method on Roche Elecsys 2010 instrument.

3.5.6.1.1 Principle

The Elecsys T₃ assay employs a competitive test principle with polyclonal antibodies specifically directed against T₃. Endogenous T₃, released by the action of 8-anilino-1-naphthalene sulfonic acid (ANS), competes with the added biotinylated T₃-derivative for the binding sites on the antibodies labeled with the ruthenium complex.

3.5.6.1.2 Reagents - working solutions

- Streptavidin-coated microparticles 0.72 mg/mL; preservative.
- Anti-T₃-Ab-Ru(bpy)₃ + contains polyclonal anti-T₃-antibody (sheep) labeled with ruthenium complex 75 ng/mL; ANS 0.8 mg/mL; phosphate buffer 100 mmol/L, pH 7.4; preservative.
- T₃-biotin contains biotinylated T₃ 3 ng/mL; ANS 0.8 mg/mL; phosphate buffer 100 mmol/L, pH 7.4; preservative.

3.5.6.1.3 Test procedure

Competition principle. Total duration of assay: 18 minutes.

- 1st incubation: 30 pL of sample and a T₃-specific antibody labeled with a ruthenium complex; bound T₃ is released from the binding proteins in the sample by ANS.
Material and Methods

- 2nd incubation: After addition of streptavidin-coated microparticles and biotinylated T₃, the still-free binding sites of the labeled antibody become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.

- The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with procell. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier.

- Results were determined via a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

3.5.6.2 Total thyroxine (T₄)

The estimation of T₄ was done by electrochemiluminance method on Roche Elecsys 2010 instrument.

3.5.6.2.1 Principle

The Elecsys T₄ assay employs a competitive test principle with an antibody specifically directed against T₄. Endogenous T₄, released by the action of 8-anilino-1-naphthalene sulfonic acid (ANS), competes with the added biotinylated T₄-derivative for the binding sites on the antibodies labeled with the ruthenium complex.

3.5.6.2.2 Reagents - working solutions

- Streptavidin-coated microparticles 0.72 mg/mL; preservative.

- Anti T₄-Ab-Ru(bpy)3+ contains Polyclonal anti-T₄-antibody (sheep) labeled with ruthenium complex 100 ng/mL; ANS 1 mg/mL; phosphate buffer 100 mmol/L, pH 7.4; preservative.

- T₄-biotin contains biotinylated T₄ ng/mL; ANS 0.8 mg/mL; phosphate buffer 100 mmol/L, pH 7.4; preservative.
3.5.6.2.3 Test procedure

Competition principle: Total duration of assay: 18 minutes.

- 1st incubation: 15 pL of sample and a T₄-specific antibody labeled with a ruthenium complex; bound 14 is released from binding proteins in the sample by ANS.

- 2nd incubation: After addition of streptavidin-coated microparticles and biotinylated T₄, the still-free binding sites of the labeled antibody become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.

- The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically, captured onto the surface of the electrode. Unbound substances were then removed with procell. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier.

- Results were determined via a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

3.5.6.3 Free triiodothyronine (FT₃)

The estimation of FT₃ was done by electrochemiluminance method on Roche Elecsys 2010 instrument.

3.5.6.3.1 Principle

In the Elecsys FT₃ test the determination of free thyroxine is made with the aid of a specific anti-T₃ antibody labeled with a ruthenium complex. The quantity of antibody used is so small (equivalent to approx. 1-2 % of the total T₃ content of a normal serum sample) that the equilibrium between bound and unbound T₃ remains virtually unaffected.

3.5.6.3.2 Reagents - working solutions

- Streptavidin-coated microparticles 0.72 mg/mL; preservative.
Material and Methods

- Anti-T₃-Ab-Ru(bpy)₃* contain polyclonal anti-T₃-antibody (sheep) labeled with ruthenium complex 50 ng/mL; phosphate buffer 100 mmol/L, pH 7.0; preservative.

- T₃-biotin : biotinylated T₃ 2.5 ng/mL; phosphate buffer 100 mmol/L, pH 7.0; preservative.

3.5.6.3.3 Test procedure

Competition principle: Total duration of assay: 18 minutes.

- 1st incubation: 15 pL of sample and a T₃-specific antibody labeled with a ruthenium complex.

- 2nd incubation: After addition of biotinylated T₃ and streptavidin-coated microparticles, the still-free binding sites of the labeled antibody become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.

- The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with procell. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier.

- Results were determined via a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

3.5.6.4 Free thyroxine (FT₄)

The estimation of FT₄ was done by electrochemiluminance method on Roche Elecsys 2010 instrument.

3.5.6.4.1 Principle

In the Elecsys FT₄ test the determination of free thyroxine is made with the aid of a specific anti-T₄ antibody labeled with a ruthenium complexa. The quantity of antibody used is so small (equivalent to approx. 1-2 % of the total T₄ content of a
normal serum sample) that the equilibrium between bound and unbound T₄ remains virtually unaffected.

3.5.6.4.2 Reagents - working solutions

- Streptavidin-coated microparticles 0.72 mg/mL; preservative.

- R1 Anti-T₄-Ab-Ru(bpy)₃* contains polyclonal anti-T₄-antibody (sheep) labeled with ruthenium complex 50 ng/mL; phosphate buffer 100 mmol/L, pH 7.0; preservative.

- R2 T₄-biotin contains biotinylated T₄ 2.5 ng/mL; phosphate buffer 100 mmol/L, pH 7.0; preservative.

3.5.6.4.3 Test procedure

Competition principle: Total duration of assay: 18 minutes.

- 1st incubation: 15 µL of sample and a T₄-specific antibody labeled with a ruthenium complex.

- 2nd incubation: After addition of biotinylated T₄ and streptavidin-coated microparticles, the still-free binding sites of the labeled antibody become occupied, with formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.

- The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with procell. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier.

- Results were determined via a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

3.5.6.5 Thyroid stimulating hormone (TSH)

The estimation of TSH was done by electrochemiluminance method on Roche Elecsys 2010 instrument.
3.5.6.5.1 Principle

The Elecsys TSH assay employs monoclonal antibodies specifically directed against human TSH. The antibodies labeled with ruthenium complex consist of a chimeric construct from human and mouse-specific components. As a result, interfering effects due to HAMA (human anti-mouse antibodies) are largely eliminated.

3.5.6.5.2 Reagents - working solutions

- Streptavidin-coated microparticles 0.72 mg/mL, preservative.
- R1 Anti TSH-Ab-biotin contains biotinylated monoclonal anti-TSH antibody (mouse) 2.0 mg/L; phosphate buffer 100 mmol/L, pH 7.2; preservative.
- R2 Anti-TSH-Ab-Ru(bpy)3+ contains monoclonal anti-TSH antibody (mouse/human) labeled with ruthenium complex 1.2 mg/L; phosphate buffer 100 mmol/L, pH 7.2; preservative.

3.5.6.5.3 Procedure

Sandwich principle: Total duration of assay: 18 minutes.

- 1st incubation: 50 μL of sample, a biotinylated monoclonal TSH specific antibody and a monoclonal TSH-specific antibody labeled with a ruthenium complex react to form a sandwich complex.
- 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with procell. Application of a voltage to the electrode then induces chemiluminescent emission which was measured by a photomultiplier.
- Results were determined via a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.
3.6. Statistical analysis

SPSS (Statistical Package for Social Sciences) software (SPSS. Version 17.0: SPSS Inc. Chicago, IL) was used for the statistical calculations. Prevalence was carried out using Microsoft Excel program. Results for continuous variable were presented as mean ± SD.

The differences between the groups were tested for significance by Student's t-test.

The relationship between variables was analyzed by Pearson's correlation or Spearman's rho correlation test. Differences and correlations were considered significant at \( p < 0.05 \). Differences and correlations were considered highly significant at \( p < 0.01 \).