MATERIALS AND METHODS
Chapter 2

MATERIALS AND METHODS
2.1. Study group

The study group consists of 129 subjects and they are categorized into four. The age group is between 40 and 65 years.

**Control group:** Control group consisted of 25 subjects out of which 14 were male and 11 were females who are considered to be normal, without any clinical manifestations.

**Non alcoholic liver cirrhosis (NALC) group:** This group consisted of 33 subjects, out of which 24 were male and 9 were females. They were clinically diagnosed as liver cirrhotic patients, who were non alcoholics.

**Alcoholic liver cirrhosis (ALC) group:** This group consisted of 33 subjects who were all males. They were clinically diagnosed with liver cirrhosis and they were chronic alcoholics.

**Hepatocellular carcinoma (HCC) group:** This group consisted of 38 subjects, out of which 28 were males and 10 were females. They were clinically diagnosed of having hepatocellular carcinoma.

2.2. Methods

The serum samples were collected from different hospitals of Bareilly city of Uttar Pradesh. The samples used were serum samples, which was used for other diagnostic purposes. After serum sample collection, different serum parameters were analysed. The parameters analysed were serum bilirubin, serum albumin, serum enzymes (aspartate, aminotransferase, alanine aminotransferase, alkaline phosphatase, γ - glutamyl transferase) and serum acute phase proteins (C–reactive proteins, transferrin, ferritin and ceruloplasmin).
All the parameters were analysed using standard kits. Bilirubin was determined by diazotized sulphanilic acid method and albumin was determined by bromo cresol green dye method. The enzymes were determined by kinetic method. The acute phase proteins, C-reactive protein, transferrin and ferritin were determined by ELISA technique and ceruloplasmin by immunoturbidimetry method.

2.2.1. Determination of serum bilirubin

Diazo Method of Pearlman and Lee, End Point

Principle:

Bilirubin reacts with diazotised sulphanilic acid in an acidic medium to form pink coloured azobilirubin with absorbance directly proportional to the bilirubin concentration.

Reagent composition:

Reagent 1: Total bilirubin reagent contains surfactant 1 %, HCl 100 mMol/L, sulphanilic acid 5 mMol/L.

Reagent 2: Sodium nitrite reagent.

Working reagent preparation: To 10 ml of reagent 1, 0.2 ml of reagent 2 was added.

Sample: Serum.

Assay procedure:

<table>
<thead>
<tr>
<th>Contents added</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>500 µl</td>
<td>500 µl</td>
<td>500 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>25 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>25 µl</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>-</td>
<td>-</td>
<td>25 µl</td>
</tr>
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</table>
The contents in each tube were mixed well and incubated for 5 minutes at 37°C and the absorbance was read at 630 nm against reagent blank.

**Calculation:**

\[
\text{Total bilirubin mg/dl} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{Concentration of standard mg/dl}
\]

Normal range: 0.1 – 1.2 mg/dl

**2.2.2. Determination of serum albumin**

BCG dye method, End Point

**Principle:**

Albumin binds with bromopressol green at pH 4.2 causing a shift in the absorbance of the yellow BCG dye. The blue green colour formed is proportional to the concentration of albumin present, which is measured photometrically at 630 nm.

**Reagent composition:**

Reagent 1: Albumin reagent: It contains bromopressol green 0.08 mMol/L. Succinate buffer (pH 4.2 ± 0.1 at 25°C) 50 mMol/L. Sodium azide: 1 g/L. Albumin standard: 3.6 g/dl

Sample: Serum

**Assay procedure:**

<table>
<thead>
<tr>
<th>Contents added</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µl</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
The contents in each tube was mixed well and kept at 37 °C for one minute. The absorbance of the standard and test were read at 630 nm against reagent blank.

**Calculation:**

\[
\text{Albumin g/dl} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{concentration of standard g/dl}
\]

Normal value: 3.5 – 5 g/dl

### 2.2.3. Determination of aspartate aminotransferase (AST/SGOT)

**Principle:**

Aspartate aminotransferase catalyses the transfer of the amino group from aspartate to α-ketoglutarate forming oxaloacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH measured at 340 nm by means of a malate dehydrogenase (MDH) coupled reaction.

\[
\begin{align*}
\text{Aspartate} + \alpha\text{-ketoglutarate} & \rightarrow \text{oxaloacetate} + \text{glutamate} \\
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ & \rightarrow \text{Malate} + \text{NAD}^+
\end{align*}
\]

**Composition:**

Reagent A: Tris 121 mMol/L, Laspaptate 362 mMol/L, malate dehydrogenase > 460 U/L, Lactate dehydrogenase > 660 U/L, Sodium hydroxide 255 mMOL/L, pH 7.8

Reagent B: NADH 1.3 mMol/L, 2 oxaloglutarate 75mMol/L, sodium hydroxide 148 mMOL/L, sodium Azide 9.5 g/L.

Working reagent: The contents of the reagent B is added into the reagent A bottle (4ml Reagent A: 1ml Reagent B). It was mixed well and stored at 2-8 °C.

Sample: Serum
Procedure:

The working reagent and the instrument were brought to 30 °C and one ml of working reagent was added into the cuvette. Into this, 100 µl of the sample was added. The content was mixed and the cuvette was inserted into the photometer. A stopwatch was switched on and after one minute the initial absorbance was recorded and after each minute for three consecutive minutes the absorbance was recorded. The difference between the consecutive absorbances were calculated and the average absorbance difference per minute was arrived at (ΔA/Min).

Calculation:

The AST concentration of the sample \[= \frac{\Delta A}{\text{Min}} \times \frac{V_t \times 10^6}{e \times l \times V_s} \text{ U/L} \]

The molar absorbance (ε) of NADH at 340 nm is 6300, the path length (l) is 1 cm, the total reaction volume (Vt) is 1.1 at 30°C, the sample volume (Vs) is 0.1 at 30°C. The above formula will give a factor (1746). When this factor is multiplied by ΔA/min, it will give the activity in U/L.

Normal value: <35 U/L for male and <31 for female.

2.2.4. Determination of alanine aminotransferase

NADH Kinetic UV

Principle:

Alanine amino transferase catalyses the reversible transfer of an amino group from alanine to α - ketoglutarate forming glutamate and pyruvate. The pyruvate produced is reduced to lactate by lactate dehydrogenase and NADH. The rate of decrease in NADH measured photometrically and is proportional to the catalytic concentration of ALT present in the sample.
L - alanine + α - ketoglutarate \[\text{ALT}\] \rightarrow \text{glutamate + pyruvate}

Pyruvate + NADH+H+ \[\text{LDH}\] \rightarrow \text{Lactate + NAD}^+

**Reagents:**

R1 buffer: It contains TRIS pH 7.8 100 mMol/L, Lactate dehydrogenase 1200 Units/L and L- alanine 500 mMol/L.

R2 substrate: It contains NADH 0.18 mMol/L and α - keto glutarate 15 mMol/L.

Working reagent: It was prepared by mixing 4 volumes of R1 buffer and 1 volume of R2 substrate.

**Procedure:**

One ml working reagent was taken in the cuvette and 100 μl of serum sample was added. It was mixed well and incubated for 1 minute. After noting the initial absorbance, absorbances were recorded every one minute for three minutes. The difference between the absorbances and the average absorbance per minute (ΔA/min) was calculated.

Calculation: ΔA/min X 1750 = IU/L of ALT. Where, 1 International Unit is the amount of enzyme that transforms 1 μMol of substrate/minute.

Normal value: < 45 U/L for male and < 34 for female.

2.2.5. Determination of alkaline phosphatase

(ALP) – AMP method

**Principle:**

Alkaline phosphatase catalyses the transfer of the phosphate group from 4-nitrophenyl phosphate to 2- amino, 2- methyl, 1- propanol (AMP), liberating 4-
nitrophenol in alkaline medium. The catalytic concentration is determined from the rate of 4- nitrophenol formation measured at 405 nm.

4-nitrophenyl phosphate + AMP $\rightarrow^ {\text{ALP}}$ AMP - phosphate + 4- nitrophenol

Composition:

Reagent A: It contains 2- amino, 2- methyl, 1- propanol, 0.4 mol/L, zinc Sulphate 1.2 mMol/L N-hydroxy ethylethelene diamine triaceticacid 2.5 mMol/L magnesium acetate 2.5 mMol/L, pH 10.4.

Reagent B: 4- nitrophenyl phosphate 60 mMol/L

Working reagent: The contents of reagent B was transferred into a reagent A bottle and was mixed gently (4 ml reagent A + 1 ml reagent B).

Sample: Serum

Procedure:

The working reagent and the instrument were brought to reaction temperature and one ml of working reagent was added into the cuvette. Into this 20 µl of the sample was added. The content was mixed and the cuvette was inserted into the photometer. A stopwatch was switched on and after one minute the initial absorbance was recorded and after each minute for three consecutive minutes the absorbance was recorded. The difference between the consecutive absorbances was calculated and the average absorbance difference per minute was arrived at ($\Delta A$/Min).

Calculation:

The ALP catalytic concentration of the sample $= \frac{\Delta A/\text{Min} \times V_t \times 10^6}{\varepsilon \times 1 \times V_s}$ U/L

The molar absorbance ($\varepsilon$) of 4- nitrophenol at 405 nm is 18450, the path length (l) is 1 cm, the total reaction volume (Vt) is 1.02, the sample volume (Vs) is...
0.02. The above formula will give a factor (2764). When this factor is multiplied by $\Delta A/min$, will give the activity of alkaline phosphatase in U/L.


2.2.6. Determination of serum $\gamma$-glutamyl transferase

Szasz method

**Principle:** $\gamma$-glutamyl para nitro anilide (GPNA) and glycyl glycine are converted by the action of $\gamma$-glutamyl transferase to para nitro aniline and L-$\gamma$-glutamyl glycine. The rate of increase in absorbance at 405 nm due to the release of para nitro aniline is directly proportional to the GGT activity.

\[
\text{GPNA} + \text{glycyl glycine} \quad \downarrow \quad \text{GGT} \\
\text{P-nitroaniline} + \text{L-$\gamma$-glutamyl glycyl glycine}
\]

**Reagents:**

1) GGT substrate.
2) GGT buffer.

**Working reagent preparation:** Add 1.1 ml of GGT buffer was added to 1 bottle of GGT substrate. It was mixed well to dissolve and kept for 15 minutes prior to use.

**Sample:** Serum

**Procedure:**

All the reagents and the sample are brought to reaction temperature of 37 °C. Into a test tube, 1 ml of working reagent was added and into this 0.1 ml of serum was added. It was mixed well and read the first absorbance of the test exactly at 60
seconds and then second, third and fourth at an interval of 30 seconds at 405 nm. The mean change of absorbance per minute \((\Delta A/\text{min})\) was determined.

**Calculations:**

Serum GGT activity \((\text{IU/L}) = \Delta A/\text{min} \times F\), where \(F = 1158\) (calculated on the basis of molar extinction coefficient for para nitro aniline and sample to total volume ratio.

Normal value: < 55 U/L for male and < 38 U/L for female.

### 2.2.7. Determination of Serum C-reactive Protein

**CRP ELISA KIT**

**Principle:**

CRP ELISA kit is based on simultaneous binding of human CRP from samples to two antibodies, one immobilized on the microtiter well plates, and the other conjugated to the enzyme horseradish peroxidase. After a washing step, chromogenic substrate is added and colors developed. The enzymatic reaction (color) is directly proportional to the amount of CRP present in the sample. Adding stopping solution terminates the reaction. Absorbance is then measured on a microtiter well ELISA reader at 450 nm and the concentration of CRP in samples and control is read off the standard curve.

**Contents:**

- Anti-human CRP coated strip plate 1001U
- CRP Std. A (0 ng/ml), or Sample Diluent, 50 ml 1002U
- CRP Std B (5 ng/ml), 0.50 ml 1003 U
- CRP Std C (10 ng/ml), 0.50 ml 1004U
- CRP Std D (25 ng/ml), 0.50 ml 1005U
CRP Std E (50 ng/ml), 0.50 ml 1006U
CRP Std F (100 ng/ml), 0.50 ml 1007U
Human CRP Control Serum 0.5 ml
Anti-hCRP-HRP Conjugate, 1 1 ml 1008U
HRP substrate, Solution A., 1 1 ml 1000SA
HRP substrate, Solution B., 1 1 ml 1000SB
Wash buffer (20X), 50 ml W B - 2 0
Stop solution, 1 0 m 1 T - 1 0

Reagents preparation: Wash buffer was diluted with deionised water in the ratio 1:20 (50 ml is diluted to 1000 ml of deionised water).

Serum dilution: The serum sample was diluted at the ratio 1:100 with the diluent (5 µl in 500 µl sample diluent).

Procedure:

All the reagents were brought to room temperature. To all the wells 200 – 300 µl wash buffer was added. It was mixed for 5 seconds and discarded. The microtitre wells were labeled and 10 µl standards and diluted samples were added into the respective wells. Into each well 100 µl of Ab-enzyme conjugate was added and mixed gently for 5 – 10 seconds. The plate was covered and incubated for 30 minutes at room temperature. The wells were washed 5 times with the wash buffer. The required amount of substrate solution A and B in the ratio 1:1 was prepared. Then 200 µl of the above substrate was added into each well. The plate was covered and incubated for 10 minutes at room temperature. Blue color developed in standards and positive wells. At this time the reaction was stopped by adding 50 µl of stop solution to all the wells and mixed gently for 5-10 seconds. The blue color turned to yellow. The absorbance was measured at 450 nm using an ELISA reader.
Calculation:

A standard curve on log-log graph paper was prepared and the reading was interpreted by plotting net absorbance values of standards against appropriate CRP concentrations. Multiply the values by 100 or the dilution factor of the samples if samples were diluted by a factor other than 1:100. From the optical density of the diluted sample in the well, the concentration of the CRP in the diluted sample was calculated from the graph. The obtained values were multiplied by the appropriate dilution factor to get the amount of CRP in serum.

Normal values: 0.0 - 8 mg/L

2.2.8. Determination of serum transferrin

Immunoenzymetric Assay

Principle:

The human transferrin assay is a two-site immunoenzymetric assay. Samples containing human transferrin are reacted in microtiter strips coated with an affinity purified capture antibody. A second HRP labeled anti-human transferrin antibody is reacted, forming a sandwich complex of solid phase antibody transferrin - HRP labeled antibody. After a wash step to remove any unbound reactants the strips are then reacted with TMB substrate, followed by the addition of a stop solution changing the color from blue to yellow. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of human transferrin present in the sample. Accurate quantitation is achieved by comparing the signal of known human transferrin standards assayed at the same time.

Reagents:

Anti-human Transferrin labeled with HRP F039
Affinity purified goat antibody in a protein matrix with preservative, 1x12 L
Goat anti-transferrin coated microtiter strips F059 12x8 well strips in a bag
with desiccant.

Human Transferrin Standards F037 human transferrin in a protein matrix
with preservative. Standards at 0, 0.125, 0.5, 2, and 8 ng/ mL.

Tetra methyl benzidine substrate F005
3,3'',5,5''Tetramethyl benzidine, 1x12 mL
Stop Solution F006
0.5N Sulfuric Acid, 1x12 mL
Wash Concentrate (20X) F004
Tris buffered saline with preservative, 1x50 mL
Sample Diluent
Distilled water
1 liter wash bottle for diluted wash solution

Preparation of Reagents:
All the reagents are brought to room temperature. The wash concentrate
was diluted to 1 liter in distilled water, and labelled and stored at 4 °C.

Procedure:
Into the respective wells 50 µL of standards, controls and samples were
added. To this, 100 µL of anti-transferrin HRP (# F039) was added into each well.
It was incubated on a rotary shaker at 180 rpm for 2 hours. The wells were washed
thoroughly for four times. To this 100 µL of TMB substrate was added and
incubated for 30 minutes. Then, 100 µL of stop solution was added and the
absorbance was read at 450/630nm with a blank solution.
Calculation:

A standard curve was constructed by taking the absorbance values of the standards on the y-axis and concentration on the x-axis. Absorbances of samples are then interpolated from this standard curve to arrive at the real value.

2.2.9. Determination of serum ferritin

DRG® Ferritin ELISA

Principle:

Anti-human-ferritin antibodies are coated onto the microwells. Ferritin, if present in diluted serum, will bind to the microwells. Washing of the microwells removes unreactive serum components. Horseradish peroxidase (HRP) conjugated anti-human ferritin immunologically bind to the bound patient ferritin forming a conjugate – ferritin – antibody complex. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450 nm. The amount of colour is directly proportional to the concentration of ferritin present in the original sample.

Contents:

Divisible microplate consisting of 12 modules of 8 wells each, coated with highly purified specific anti-human-ferritin antibodies (rabbit, polyclonal). Ferritin-Calibrators (A-F) in a PBS/BSA matrix (NaN₃ <0,1% (w/w)) containing ferritin: 0; 15; 50; 150; 500 and 1500 ng/ml.

Ferritin Controls in a PBS/BSA matrix (NaN₃ <0,1% (w/w)) positive (1) and negative (2), for the respective concentrations see the enclosed package insert.

Sample buffer (Tris, NaN₃ <0,1% (w/w)), yellow.
Enzyme conjugate solution (PBS, PROCLIN 300 <0,5% (v/v)), (light red) containing polyclonal rabbit anti-human ferritin; labelled with horseradish peroxidase.

TMB substrate solution.
Stop solution (1 M hydrochloric acid).
Wash solution (PBS, NaN3 <0,1% (w/w), concentrate (50x))

Preparation of reagents:
Preparation of wash solution: The buffered wash concentrate (50 X) of each vial was diluted with deionized water to a final volume of 1000 ml prior to use.

Procedure:
Into the respective wells 25 μl of calibrators, controls and patient samples in duplicate were added. Into each well 100 μl sample buffer was added. It was incubated for 30 minutes at room temperature. The contents of the microwells were washed 3 times with 300 μl of wash solution. Into each well 100 μl of enzyme conjugate was added and incubated for 15 minutes at room temperature. The contents of the microwells were discarded and washed 3 times with 300 μl of wash solution. Into each well 100 μl of TMB substrate solution was added and incubated for 15 minutes at room temperature. To each well 100 μl of stop solution was added and incubated for 5 minutes at room temperature. The optical density was read at 450 nm and the results were calculated.

Calculation:
A standard curve was prepared and the concentration of the sample was calculated.

Normal value:
Female: 20 – 50 years 22 – 112 ng/ml; Female: 65 – 90 years 13 – 651 ng/ml; Male: 20 – 50 years 34 – 310 ng/ml; Male: 65 – 87 years 4 – 665 ng/ml.
2.2.10. Determination of serum ceruloplasmin

Immunoturbidimetry

Principle:

The determination of human ceruloplasmin is based on the reaction between ceruloplasmin as antigen and the specific antiserum as antibody. This reaction forms an insoluble complex producing a turbidity, which is measured spectrophotometrically at 340 nm.

Reagents:

Reagent 1: TRIS/PEG buffer pH 7.5

Reagent 2: Antiserum Anti-Ceruloplasmin

Optional: 101-0485 General proteins calibrator

Working reagent preparation:

Reagent 2 was diluted with the buffer solution. The dilution depends on the analyser (Inquire). It is stable, at 2-8°C, up to the expiration date.

Calibration curve: Prepare dilutions of the General Proteins calibrator using 9 g/L as diluent.

The Ceruloplasmin calibrator concentration was multiplied by the corresponding dilution factor indicated in the table to obtain the Ceruloplasmin concentration of the different calibrators.

Sample: Serum.

Procedure:

All the reagents were brought to 37 °C before starting the experiment.

Preparation of working reagent:
The Antiserum Anti-ceruloplasmin (Reagent 2) was diluted with the buffer solution (Reagent 1) in the ratio 1:41. The sample and the controls were diluted with saline solution (0.9 % NaCl) at the ratio of 1:21. The following contents were pipetted into the cuvette.

<table>
<thead>
<tr>
<th>Contents pipetted into the cuvette</th>
<th>Blank</th>
<th>Calibrator</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (9g/L)</td>
<td>50 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calibrator</td>
<td>-</td>
<td>50 μl</td>
<td>-</td>
</tr>
<tr>
<td>Dil. Sample</td>
<td>-</td>
<td>-</td>
<td>50 μl</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>

The contents were mixed and kept for ten minutes and read the absorbance (A) against the blank at 340 nm.

**Calculation:**

The absorbance for each calibrator was calculated and the values were plotted against the concentration in a calibration curve. Ceruloplasmin concentration in the sample was calculated by interpolation of its A value on the calibration curve. Normal value: Between 15 – 60 mg/dL.

**Statistical analysis:**

All the values were expressed as mean±SD. The statistical analysis (One way ANOVA) was done using the SPSS 12 software package. Statistical significance was set at P<0.05.