MATERIALS AND METHODS

RESEARCH DESIGN

Prospective, Observational, Case Control research Study was carried out in a Single Centre.

TIME SPAN OF RESEARCH WORK

The research work was completed within a span of two years starting from January, 2010 up to January, 2012.
SAMPLING DESIGN

➢ PLACE OF STUDY

The study was conducted in the Department of Gastroenterology and Hepatology, at Delhi Heart Institute and Research Centre, located in Bathinda (Punjab). It is a super-speciality centre.

➢ SIZE OF SAMPLE

Sample comprised of total 200 participants, which were consisted of 100 patients of liver disease and another 100 healthy individuals as controls.

➢ SAMPLING METHOD

Proportional Stratified Sampling Method was employed to recruit study sample from the population. The study population comprised of the patients suffering from liver diseases, who visited the department and also those who were admitted in the ward of gastroenterology.

PATIENTS STRATIFICATION CRITERIA

Patients were stratified on the basis of following criteria as given below:

1. Serum Aminotransferase Level

2. Presence of Ascites

Cut off point for Serum Aminotransferase, was taken as 300 IU.

1. GROUP 1, Patients, with SGPT level >300 IU, along with SGPT value > SGOT value and without the presence of Ascites, were diagnosed as viral hepatitis and were kept in Group 1 (Pratt & Kaplan, 2008). This group consisted of 33 patients.

2. GROUP 2, Patients, who had SGPT level < 300 IU, along with SGOT value > SGPT value and were without the presence of Ascites, were diagnosed as Non Ascites cirrhosis (compensated cirrhosis). These patients were taken in Group 2 (Pratt & Kaplan, 2008). This group consisted of 30 patients.
3. **GROUP 3, Patients with SGPT level < 300 IU, along with SGOT value > SGPT value and presence of Ascites (as determined by clinical examination), were diagnosed as Ascites cirrhosis (Decompensated cirrhosis) and were kept in Group 3 (Pratt & Kaplan, 2008).** This group consisted of 37 patients.

A similar research design was adopted by (Gotzberger et al., 2008), in which, 81 patients of liver diseases and 78 healthy controls were taken. The patients were stratified into Group -1 (n=21) Ascites cirrhosis patients

Group-2 (n=25) Non Ascites cirrhosis patients

Group – 3 (n=35) Fatty liver

Group-4 (n=78) Healthy controls

**PATIENTS SELECTION CRITERIA**

Patients were diagnosed to be suffering from liver disease on the basis of case history, General physical examination and diagnosis was confirmed by laboratory estimation of biochemical markers of liver and renal functions.

The following criteria were adopted for the inclusion and exclusion of patients, as under:

**PATIENTS INCLUSION CRITERIA**

1. Patients with acute viral hepatitis .
2. Patients with cirrhosis of liver.
3. Patients of 18 years and above.
4. Patients of both gender were eligible.

**PATIENTS EXCLUSION CRITERIA**

1. Patients of cholestatic liver disorder, liver malignancy and non alcoholic fatty liver disorders.
2. Patients with history of any other systemic, metabolic or endocrine disease.
3. Patients with history of AIDS.
4. Pregnant women.
5. Patients who were involved in any other interventional clinical trial.

CRITERIA FOR SELECTION OF CONTROLS
Participants, equal to the number of patients,(100 cases : 100 controls), were taken as Controls. The controls were Age and sex matched. The controls were selected from amongst the individuals who accompanied patients and also from the volunteers in the hospital. The controls were Age and Sex matched. Controls constituted the GROUP 4, (n=100).

CONTROLS INCLUSION CRITERIA
1. Absence of history of any systemic, metabolic or endocrine disease.
2. Absence of any liver disease.
3. Controls were from the hospital population of healthy individuals.
4. Controls were Age and Sex matched.

CONTROLS EXCLUSION CRITERIA
1. Presence of history of blood transfusion.
3. Presence of history of involvement in any other study.

STUDY VARIABLES
Liver function parameters were the Independent variables, whereas, Renal function parameters were taken as Dependant variables.

INDEPENDENT VARIABLES
Serum Bilirubin, Serum Glutamate-Pyruvate Transaminase (SGPT), Serum Glutamate-Oxaloacetate Transaminase (SGOT), Serum Albumin, International Normalised ratio(INR), Haemoglobin concentration.

DEPENDENT VARIABLES
Estimated Glomerular Filtration rate (by MDRD Equation), Serum Creatinine, Blood Urea Nitrogen, BUN/Creatinine ratio, Serum Sodium concentration, Serum Potassium concentration.

DATA COLLECTION METHODS AND INSTRUMENTS
Primary data were collected from patients admitted in the ward of Gastroenterology, and from individuals, who escorted the patients as well as from the volunteers.

METHODS AND INSTRUMENTS
1. Schedules were used for the collection of demographic data of participants.
2. Structured observation method was utilized to collect data regarding clinical signs and symptoms of patients. These data point were recorded on pre-structured proforma.
3. Laboratory estimation of biochemical parameters of liver and renal functions, were undertaken and data were recorded on Pre-Structured Proforma.

The liver and renal function parameters were estimated by following biochemical methods.

METHODS OF ESTIMATION OF BIOCHEMICAL PARAMETERS
Blood samples were aspirated from vein in cubital fossa. Serum from each sample separated and following methods were used to collect data of biochemical parameters of liver and kidney functions (Singh, 2006).

1. Estimation of Serum Bilirubin: Serum Bilirubin is converted into purple compound of Azobilirubin when the bilirubin is treated with Diazotised sulphinilic acid (Ehrlich’s reagent, where sulphanilic acid is converted into its highly reactive diazonium salts by treatment with nitrous acid produced from reaction between sodium nitrite and
hydrochloric acid). This reaction was introduced by Van den Bergh in 1913. He also observed the presence of two types of reactions as fast and delayed.

PRINCIPLE: (Jendrassik and Groff, 1938 method)
Serum is treated with Diazotized sulphanilic acid and this forms Azobilirubin complex. The direct bilirubin (conjugated) is a polar and water soluble, due to presence of glucuronic acid moiety (bilirubin diglucuronides) and reacts with diazo reagents fast (within one minute), whereas, the indirect bilirubin is unconjugated, non polar, non soluble in water and is present in serum as a bilirubin-albumin complex, reacts with diazo reagent in the presence of an accelerator like caffeine- sodium benzoate reagent( 10 minutes, delayed reaction).

The azobilirubin, so produced is purple in colour in acidic medium. This colour is changed into blue by the addition of alkaline tartarate solution. The absorbance is read at 600 nm and colour is stable for 30 minutes.

The specimen was first reacted with diazo reagents without the presence of accelerator and absorbance is read for colour produced. This represented direct bilirubin. Thereafter, the specimen was reacted with diazo salt in presence of accelerator and now, the absorbance represented the total bilirubin. The difference of the two values showed the indirect bilirubin value (Ochei & Kolhatkar, 2000).

2. Estimation of Serum Albumin: It was done by Bromocresol Green dye (BCG) method).
PRINCIPLE: In this method, serum albumin binds to Bromocresol green dye, specifically, under acidic conditions. This reaction produces a green coloured, albumin-BCG complex. The absorbance is read at 640 nm( red filter). The intensity of the colour of complex produced is directly proportional to the amount of albumin present in serum (Chawla, 2008).

3. Estimation of Serum Aminotransferases (SGPT and SGOT): It was done by the method of Reitman and Frankel.
Serum aminotransferases are the Transferase group of enzymes that catalyse the transfer of amino group from an alpha amino acid to alpha keto acid, resulting into production of new amino acid and a new keto acid. These enzymes are also called Transaminases. This step is employed in the catabolism of amino acids. 

All the naturally occurring amino acids undergo transamination by this process. Large number of transaminases are known that require alpha keto glutaric acid or oxaloacetic acid or pyruvic acid as amino group acceptor.

Two diagnostically important transaminases are present in serum, namely, Glutamate pyruvate transaminase, SGPT (also called alanine transaminase, ALT) and Glutamate oxaloacetate transaminase, SGOT (also called aspartate transaminase, AST). The SGOT is abundantly found in liver, kidney, cardiac muscles and in small amount in brain, pancreas and lungs, whereas, the SGPT is mainly found in liver. This forms the basis to detect liver injury.

**PRINCIPLE:** In this method, to determine SGPT (ALT), the serum is treated with alanine and alpha ketoglutarate. These two compounds act as substrates, whereas, to determine, SGOT (AST), the serum is treated with aspartate and alpha ketoglutarate as substrates.

Two keto acids namely, pyruvate and oxaloacetate are produced in these reactions respectively. These new keto acids are treated with 2,4-dinitrophenylhydrazine.

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\text{Aspartate} + \text{alpha Ketoglutarate} \xrightarrow{\text{AST}} \text{Glutamate} + \text{Oxaloacetate}
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\text{Oxaloacetate} + 2,4 \text{ dinitrophenyl hydrazine} \xrightarrow{\text{Alkaline medium}} 2,4 \text{ dinitrophenylhydrazone} \text{ (brown)}
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The resultant compound, 2,4dinitrophenylhydrazone, formed is of brown colour. The absorbance is read at 520nm (green filter) (Chawla, 2008).
4. **Estimation of Blood Urea**: It was estimated by urease enzymatic method as described by Nessler.

PRINCIPLE: In this method, urea is converted to ammonia by the action of enzyme urease. The ammonia so produced reacts with Nessler’s reagent (potassium mercuric iodide). The brown colour compound is produced, whose absorbance is read at 520 nm (Sood, 1999).

5. **Estimation of Serum Creatinine**: It was estimated by Jaffe’s alkaline picrate method.

PRINCIPLE: Serum is diluted with distilled water and proteins are precipitated by reacting with tungstic acid. The alkaline picrate is added to the protein free filtrate. Creatinine reacts with picric acid in alkaline medium and it forms a creatinine picrate complex. This has red colour. It is read at 520 nm. The intensity of colour produced, is proportional to the amount of creatinine in serum (Sood, 1999).

6. **Estimation of Serum Electrolytes**: By Flame Photometry method.

PRINCIPLE: In this method, for estimation of serum sodium and potassium, the emission flame photometry is used. The diluted serum is sprayed as fine droplets into the flame. The flame gets coloured due to emission of sodium or potassium ions. The amount of light emitted is dependent upon the strength of metallic ions in the serum (Chawla, 2008).

7. **Estimation of Haemoglobin**: By acid Haematinic method of Sahli.

PRINCIPLE: Haemoglobin is treated with N/10 hydrochloric acid. It gets converted into acid haematin. The brown colour of the compound is matched visually by comparing with a comparator in the Sahli’s haemoglobinometer (Ochei & Kolhatkar, 2000).
Urine analysis: random urine sample collected in dry, sterile container. It was treated with sulphasalicylic acid to detect protein. Urine sample centrifuged and urinary sediment examined microscopically. Ascites fluid examined microscopically, to detect PMN cell count.

DEFINITIONS

1. Serum creatinine concentration of > 1.5mg/dl was considered as a cut off to decide renal dysfunction (Salerno et al., 2007).

2. Jaundice: It is the hallmark symptom of liver disease and is clinically noticeable at serum bilirubin level >3 mg/dl (Ghany et al., 2008).

3. Ascites: it is the accumulation of fluid in the peritoneal cavity. It was clinically observed as an increase in girth of abdomen. Diagnosis was made by physical examination as patients had bulging flanks and shifting dullness of abdominal fluid (Mailliard & Michael, 2008).

4. Spontaneous Bacterial Peritonitis: It was confirmed by the presence of Polymorphonuclear Neutrophil count > 250/mm3 (by laboratory analysis of ascites fluid).

5. Acute Tubular Necrosis: This disorder was confirmed by the presence of renal tubule epithelial cell casts as were seen in examination of urinary sediments.

REFERANCE RANGE AND CUT OFF POINTS

1. eGFR
Glomerular filtration rate was estimated by MDRD equation, utilizing serum creatinine, age, sex, race as variables.
Reference range (90-120 ml/min/1.73m2)
Cut off point (56 ml/min/1.73m2) (Bellomo et al., 2004; Peter, 2011).
2. Serum Creatinine
Reference range (0.6-1.2 and 0.5-1 mg/dl for male, female).
cut off point (1.5 mg/dl) (Fernandez, 1995).

3. BUN
Reference range (10-20 mg/dl)
Cut off point > 20 mg/dl (Ochei & Kolhatkar, 2000).

4. BUN/CREATININE Ratio
Cut off point 20 mg.dl (Chertow, 2008).

5. Serum Bilirubin
Reference range (0.2-1.2mg/dl)
Cut off point 1.2 mg/dl (Sood, 1999).

6. Serum Albumin
Reference range (3.5-5 mg/dl).
Cut off point 3.5 mg/dl (Sood, 1999).

7. Serum Sodium
Reference range (135-145 meq/L)
Cut off point 135 meq/L (Sood, 1999).

8. INR
Reference range (0.8-1.2)
Cut off point 1.2 (Sood, 1999).

9. Serum Aminotransferases
Reference range for SGPT (up to 45 IU)
For SGOT (up to 40 IU)
Cut off point SGPT, SGOT 300 IU
10. Haemoglobin
Reference range (15±2.5 and 14±2.5 mg/dl in male and female)
Cut off point 13 mg/dl (Ochei & Kolhatkar, 2000).

11. Serum Potassium
Reference range (3.5 - 5 meq/L)
Cut off point 3.5 meq/L (Sood, 1999).

STATISTICAL DESIGN
The following statistical techniques were used for analysis of data.
1. Classification and Tabulation of data
The data, that pertained to demographic characteristics, clinical signs/symptoms, laboratory results of Random urine sample and Ascites fluid were considered as qualitative data. The data were expressed in terms of percentage (n%), and presented in the form of Tables.

2. Descriptive Statistics
The data, that pertained to independent and dependent variables, were expressed in terms of Mean and Standard Deviation, Except, the data that related to variables as Serum Bilirubin, SGPT, SGOT and Blood Urea Nitrogen, which were expressed in terms of Mean and 95% C. I. after correction by Log 10 transformation and back
transformation, (as homogeneity assumption in one way Anova was not met in above each variable in four groups).

3. One way Anova was used to compare mean of independent and dependent variables in four groups.

4. Tukey’s HSD post hoc test was used to find out group with significant difference.

5. Two samples Z-test was utilized to compare mean Age of patients with controls.

6. Pearson’s coefficient of correlation was used to observe the direction and magnitude of correlation between independent and dependent variables.

7. One sample t-test was used to compare mean of a variable in a Group with hypothetical cut off.

8. p value of 0.05 was implied as significant for all statistical tests.

9. Tukey’s HSD critical value (Q0.05 = 3.63), df (within196), for four treatment groups, was implied as significant.

STATISTICAL SOFTWARES USED FOR ANALYSIS
1. Graph pad software
2. Stat pages.org
3. Microsoft exel 2003