INVESTIGATION ON
ENZYMES AND ACUTE PHASE PROTEINS IN LIVER DISEASES

Thesis submitted to
The University of Calicut
in partial fulfillment of the requirements for the award of the degree of

Doctor of Philosophy in Biochemistry

by

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CERTIFICATE

This is to certify that the thesis entitled ***"Investigation on enzymes and acute phase proteins in liver diseases"*** is a bonafide work of **Mrs.Mini,K.**, conducted in the Department of Life Sciences, started under the guidance of Dr.V.K.Sasidharan (late) Prof. of Biochemistry and former Head of the Department. After his demise, the guideship was transferred to me, and the preparation of the manuscript for the thesis was completed under my supervision.

This thesis has not previously formed the basis for award of any other similar title of any other university or society.

16/7/08

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DECLARATION

This thesis entitled "***Investigation on enzymes and Acute phase proteins in liver diseases***" is being submitted by me to the University of Calicut., in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Biochemistry under the Faculty of Science.

This thesis is the result of my work carried out in the Department of Life Sciences under the guidance and supervision of Late Dr.V.K.Sasidharan, Former Professor of Biochemistry and Head of the Department. Due to his demise, my guideship was transferred to Dr.Fathimathu Zuhara, K., Reader in Microbiology and Head of the Department of Life Sciences, and the preparation of the manuscript for the thesis was completed under her supervision.

This thesis or any part thereof has not been submitted for any other degree, diploma or associateship.

Mini,K

I dedicate this to my parents

Late Mr. T. Govindan Nair and

Mrs. Kalarikal Sreedeviamma

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Mini, K

CONTENTS

ABBREVIATIONS

µmol	Micromol
AFP	Alpha feto protein
ALP	Alkaline phosphatase
AlT	Alanine aminotransferase
Apo Cp	Apoceruloplasmin
APP	Acute phase proteins
APR	Acute phase reactants
Asn	Asparagine
AsT	Aspartate aminotransferase
ATP	Adenosine tri phosphate
CRP	C-reactive protein
Da	Dalton
GGT	Gamma glutamyltransferase
gm	Gram
h	Hour
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
IL	Interleukin
IU	International units
Kd	Kilodalton
L	Litre
ml	Milliliter
mRNA	Messenger RNA
NAFLD	Nonalcoholic fatty liver disease
NO	Nitric oxide
RNase	Ribonuclease
ROS	Reactive oxygen species
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UDP	Uridine di phosphate

Chapter 1

INTRODUCTION

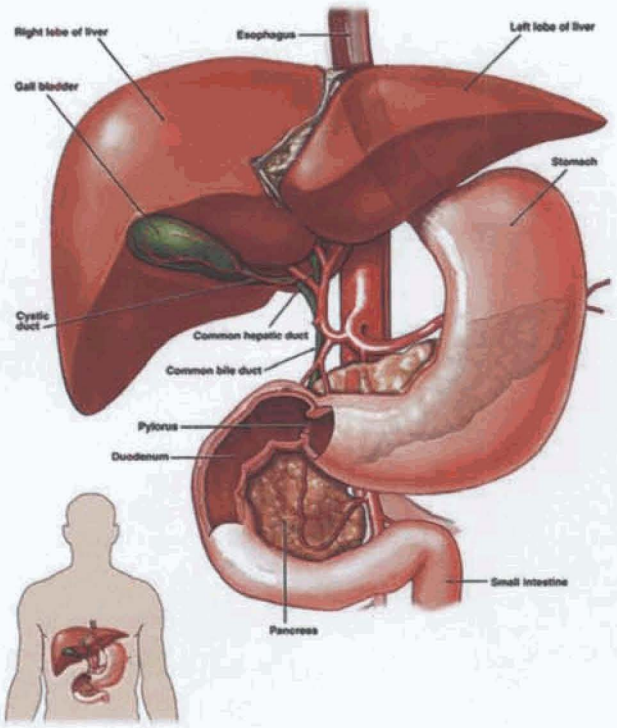
1.1. Liver

The normal liver occupies the right upper quadrant, extending from the fifth inter costal space in the mid clavicular line down to the right costal margin. The lower margin descends below the costal margin during inspiration. The average weight of liver is 1800 gm in men and 1400 gm in women (Furbank, 1967). The liver is the second largest and the heaviest organ in the body and serves a key role in critical metabolic pathways and synthetic functions. It is strategically situated to perform these diverse metabolic functions by being the first organ to receive a nutrient enriched blood supply from the portal system. It is first visible in the developing embryo in the fourth week of pregnancy. As the foetus develops, the liver divides into two sections, the right and the left lobes and ultimately the right lobe will be six times bigger than the left. The right and the left lobes are further divided into a total of eight segments in accordance with subdivisions of the hepatic portal veins. The segments each have their own hepatic artery branch and biliary tree. Each segment is made up of multiple smaller units known as lobules comprised of a central vein, radiating sinusoids separated from each other by single liver cell (hepatocytes) plates and peripheral portal racks. The functional unit of the liver is the hepatic acinus, which is anatomically almost the reverse of the hepatic lobule. The acinus is divided into 3 zones on the basis of the distance from the supplying vessels.

Liver contains hepatocytes or liver cells, a porous lining tissue of macrophages or Kupffer cells derived from blood monocytes, stellate cells found in the space of Disse and endothelial cells lining the hepatic sinusoids. Approximately 15 % of the liver is composed of cells other than hepatocytes (Sleisenger and Fordtran, 1993).

The liver has high blood flow and low vascular resistance. About 1050 ml of blood flow from the portal vein into the liver sinusoids each minute and an

additional 3000 ml flow into the sinusoids from the hepatic artery, the total averaging about 1350 ml per minute. This amounts to 27 % of the resting cardiac out put. The liver is a large, chemically reactant pool of cells that have a high rate of metabolism, sharing substrates and energy from one metabolic system to another, processing and synthesizing multiple substances that are transported to other areas of the body, and performing a myriad of other metabolic functions.



In carbohydrate metabolism, liver performs 1) storage of large amounts of glycogen (Radziuk *et al.*, 1993) 2) conversion of galactose and fructose to glucose, 3) gluconeogenesis (Pilkis and Granner, 1992) and 4) formation of many clinical compounds from intermediate products of carbohydrate metabolism (Felber and Golay, 1995).

Certain aspects of fat metabolism occur mainly in the liver. Liver is involved in the 1) oxidation of fatty acids to supply energy for other body functions, 2) synthesis of large quantities of cholestereol, phospholipids and most lipoproteins and 3) synthesis of fats from proteins and carbohydrates. The body cannot dispense without the services of the liver in protein metabolism for more than a few days without death ensuing. The most important functions of the liver in protein metabolism are 1) deamination of amino acids, 2) formation of urea for removal of ammonia from the body fluids, 3) formation of plasma proteins and 4) inter

conversions of the various amino acids and synthesis of other compounds from other amino acids (Guyton and Hall, 2000).

Essentially all the plasma proteins with the exception of part of the γ-globulins are formed by the hepatic cells. This accounts for about 90 % of all the plasma proteins. The liver can form plasma proteins at a maximum rate of 15 – 50 gm/day. Therefore, after loss of as much as one half of the plasma proteins from the body, they can be replenished in one or two weeks. It is particularly interesting that plasma protein depletion causes rapid mitosis of the hepatic cells and growth of the liver to a larger size. These effects are coupled with rapid output of plasma proteins until the plasma concentrations returns to normal.

Liver can synthesize all non essential amino acids. It has a particular propensity for storing vitamins and has been long known as an excellent source of certain vitamins in treating patients. The vitamins stored in greatest quantity in the liver is vit A, but large quantities of vit D and vit B12 are normally stored as well. Liver stores iron in the form of ferritin. When the iron in the circulating body fluid reaches a low level, the ferritin releases the iron. The apoferritin-ferritin system acts as a blood iron buffer as well as an iron storage medium. Coagulation factors like fibrinogen, prothrombin, accelerator globulin, factor VII and several other important factors are synthesized in liver.

The liver removes or excretes drugs, hormones and other substances. The active chemical medium of the liver is well known for its ability to detoxify or excrete into the bile, many drugs including sulphonamides, penicillin, ampicillin and erythromycin. Several of the hormones secreted by the endocrine glands, are either chemically altered or excreted by the liver. Liver damage can often lead to excess accumulation of one or more of these hormones in the body fluids and therefore can

cause over activity of the hormonal system. Bilirubin, the major end product of hemoglobin degradation is excreted in the bile by liver (Guyton and Hall, 2000).

1.2. Liver diseases

Liver is so complex and is susceptible to a wide variety of adverse effects caused by excess of alcohol or drugs, infections such as viral hepatitis, cancer and other metabolic disorders. But the liver is also resilient. It has the remarkable ability to regenerate itself following injury or inflammation and it has nutrient reserves, which can be tapped when it is damaged.

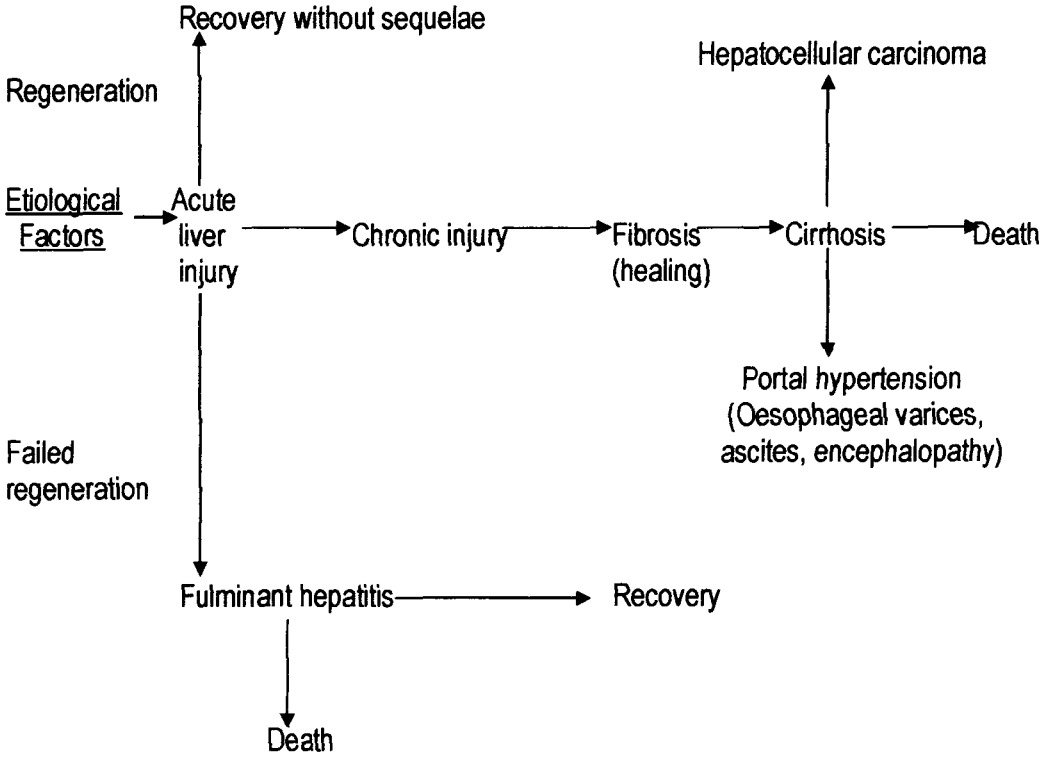
Classification of liver diseases based on etiology

Viral	**Toxic or drug induced**	**Autoimmune**
Hepatitis A, B, C, D, E.	Alcohol	Autoimmune chronic active hepatitis
Epstein Barr virus	Drugs	Primary biliary cirrhosis
Cytomegalo virus	Poisons	Vascular
Herpes simplex	**Biliary tract obstruction**	Bud-chiari syndrome
Exotic viruses	Tumors	Portal vein thrombosis
Metabolic	Strinctures	**Neoplastic**
Haemochromatosis	Gall stones	Primary malignant
Weilson's disease	Sclorising cholangitis	Benign
The hereditary hyper bilirubinaemia	Primary or secondary biliary atresia	Secondary
A 1 anti trypsin deficiency	**Miscellaneous**	**Helminthic**
Cystic fibrosis	Polysystic liver diseases	Ascariasis
Hepatic porphyria	Congenital hepatic fibrosis	Toxocariasis
Bacterial/spirochaetal	Amyloid	Clonorchis
Leptospirosis	**Protozoan**	Schistosomiasis
Tuberculosis	Kala – azar (visceral leishmaniasis	**Cryptogenic**
Pyogenic liver abscess	Amoebiasis, Malaria	

When a liver is sieged from viral hepatitis, its liver cells are damaged or destroyed. This type of injury can be initially tolerated and resisted due to the liver's ability to regenerate and compensate for the damage. This phase of liver disease is called compensated liver disease because the liver is able to continue all

its functions. When the liver begins to lose the battle, and when it is not able to regenerate liver tissue and its filtering and storing abilities are damaged by scar tissue, it reaches the end stage of liver disease called decompensated liver disease as the liver cannot compensate for the ongoing damage.

Liver disease progression pathway



Liver inflammation refers to the presence of special cells called inflammatory cells in the liver. Chronic inflammation is inflammation that persists over a long period of time. It leads to changes in liver structure, slowed blood circulation, and the death of liver cells (necrosis). Chronic inflammation eventually causes scar tissue formation, a condition known as fibrosis. Fibrosis is the harmful out come of chronic inflammation. When fibrosis become wide spread and progresses to the point that the internal structure of the liver has become abnormal, fibrosis progresses

to cirrhosis. Cirrhosis is the result of long term liver damage caused by chronic inflammation and liver cell death. The most common causes include viral hepatitis, excessive intake of alcohol, inherited diseases and hemochromatosis. Cirrhosis is accompanied by a reduction in blood supply to the liver. The loss of healthy liver tissue and reduced blood supply can lead to abnormalities in liver function. Clinical manifestations of liver disease include jaundice, portal hypertension, abnormal hepatorenal function, altered drug metabolism, nutritional and metabolic abnormalities, disordered homeostasis and the release of enzymes into various body fluids (Carl *et al.*, 2006).

1.2.1. Cirrhosis

The term cirrhosis was first used by Rene Laennec (1781-1826), to describe the abnormal liver colour of individuals with alcohol induced liver disease. The word cirrhosis comes from the Greek word Kirrhos, the name for yellowish colour. Normal functioning of the liver depends on its proper organization. Cirrhosis is a diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules (Blaker *et al.*, 2001). It is the final pathologic and clinical expression of a wide variety of chronic liver diseases (Pan *et al.*, 2004). The major complications reported to be associated with cirrhosis are jaundice (Gubernick *et al.*, 2000), infections, portal hypertension (Bilbao *et al.*, 2002), variceal and gastrointestinal bleeding (Odelowo *et al.*, 2002). Liver failure in cirrhosis is assessed by features such as ascites (Aalami *et al.*, 2000), encephalopathy (Butterworth, 2000), low serum albumin and prothrombin deficiency not corrected by vitamin K (Bustamante *et al.*, 1999).

Cirrhosis can be irreversible and life threatening. It is a public health concern because of its association with mortality and morbidity. The only available and definitive treatment is liver transplantation. Cirrhosis is however reversible in

most cases. Classically cirrhosis has been classified as 1) micro nodular, 2) macro nodular and 3) mixed, based on the histology and gross appearance of the liver.

The earliest abnormalities found to develop in cirrhosis are 1) fall in platelet count (Giannini *et al.*, 2003) 2) increase in prothrombin time, 3) decrease in the albumin to globulin ratio to less than one (Luo *et al.*, 2002) and 4) increase in the Aspartate amino transferase/Alanine amino transferase (AsT/AlT) activity ratio to greater than one (Luo *et al.*, 2002; Giannini *et al.*, 2003).

Women are more susceptible to liver damage than men. They are likely to develop at an earlier stage. When it is severe, it leads to more complications (Wong and Blendis, 2001). Liver function is usually impaired in patients with cirrhosis and because cirrhotic livers are less able to regenerate, it is important to stimulate both the regeneration and function of the remnant cirrhotic liver after hepatectomy (Burroughs *et al*, 2004).

1.2.2. Alcoholic liver cirrhosis

Alcohol abuse is a leading cause of morbidity and mortality throughout the world. Alcohol affects many organ systems of the body but perhaps most notably affected are the central nervous system and the liver. Almost all ingested alcohol is metabolized in the liver and excessive alcohol use can lead to acute and chronic liver disease. The three alcohol abuse conditions are fatty liver, hepatitis and cirrhosis (Howard, 1998). Ethanol is the most common causes of cirrhosis in the US. Genetics may play a role in the development of alcoholic liver disease. Risk factors for developing alcoholic liver disease include 1) duration and magnitude of alcohol ingestion. The risk dosage is 80 gm of alcohol per day. Daily drinking appears to be riskier than intermittent drinking (Lelbach, 1975). 2) Gender: In women there is a greater likelihood of progression of cirrhosis because of reduced activities of alcohol dehydrogenase in gastric mucosa leading to

increased blood levels of alcohol (Svikis and Reid-Quinones, 2003). 3) Hepatitis B or C infection or both may increase the severity in patients who drink heavily (Mendenhall *et al.*, 1991). 4) Genetic factors: Patterns of alcohol drinking behaviour is inherited (Whitefield, 1999; Whitefield *et al.*, 2004). 5) Nutritional status: Protein calorie malnutrition is extremely common in alcoholics. There is evidence for an immunological component in alcoholic liver disease (French, 2002; Ishii *et al.*, 1993) and there is modification of liver proteins by ethanol metabolites involved in the pathogenesis (Teare *et al.*, 1993). Alcohol is metabolised to acetaldehyde by alcohol dehydrogenase (Tanaka *et al.*, 1996) and then to acetate by acetaldehyde dehydrogenase. Genetic pleomorphism of the enzyme systems that metabolize alcohol, leading to different rates of alcohol elimination, also contributes to the individual's susceptibility to alcohol damage. Alcoholics with decreased acetaldehyde dehydrogenase activity develop alcoholic liver disease at a lower cumulative intake than others (Wong and Blendis, 2001).

Acetaldehyde formed from alcohol is toxic to different tissues, especially liver. Alcohol induced cytochrome P2E1 that leads to lipid peroxidation and low glutathione level (Nicholas *et al.*, 2006). Raised serum lipid peroxide concentrations can be found during acute inflammatory liver disease. Acute changes in liver function, reflected by high bilirubin concentrations seems to be more important for intravascular liberation of lipid peroxides than existence of specific etiologic factors or of severe long lasting global liver damage (Southorn and Powis, 1988). Consumption of alcohol increases the gut permeability to endotoxins, which induces the Kupffer cells to release tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), leading to liver inflammation (Abbas *et al.*, 1994; Male *et al.*, 1996).

Alcohol abuse can lead to the accumulation of fat within the hepatocytes, the predominant cell type in the liver. A similar condition can also be seen in some

obese people who are not alcohol abusers. Fatty liver is reversible if the patients stop drinking alcohol. However, fatty liver can lead to steatohepatitis (Howard, 1998). Fatty liver is the most frequent hepatic abnormality found in alcoholics. It is a toxic manifestation of ethanol ingestion appearing within 3–7 days of excess alcohol intake. Metabolic changes associated with ethanol ingestion result in increased triglyceride synthesis, decreased lipid oxidation and impaired secretion of the liver. This results in the accumulation of triglycerides in the hepatocytes mainly in the terminal hepatic venular zone. Fatty liver may occur alone or be part of the picture of alcoholic hepatitis or cirrhosis (Reynard *et al.*, 2002).

In some clinically important conditions such as hepatitic fibrosis, cirrhosis and fatty liver, the collagen content has been reported to be abnormal (Brandao *et al.*, 2006). Collagen deposition is a complex process that depends on synthesis in hepatic stellate cells and degradation by collagenases. Excessive deposition of collagen could occur during an imbalance in its metabolism. Alcohol intoxication activate hepatic stellate cells and Kupffer cells to secrete reactive oxygen species, that induce the production of Transforming Growth Factor-β (TGF–β) all of which induce the fibrogenic process. TGF-β and IL–6 upregulate the expression of type–1 collagen genes (Purohit and Brenner 2006). Reactive oxygen species (ROS) can inactivate enzymes containing sulphydryl groups, especially collagenases and proteases responsible for collagen degradation, which results in accumulation of collagen in liver (McCullough, 2006).

1.2.3. Non alcoholic liver cirrhosis

Nonalcoholic liver cirrhosis is mainly due to nonalcoholic fatty liver disease (NAFLD). The pathophysiology of NAFLD is complex and available data suggests that environmental factors such as exercise and toxins are likely to be important in its causation (Cotrim *et al.*, 1999). Nonalcoholic fatty liver disease is an increasingly recognized form of chronic liver disease. It encompasses a spectrum of

conditions associated with lipid deposition in hepatocytes. It ranges from steatosis (simple fatty liver) to nonalcoholic steatohepatitis and advanced to fibrosis and cirrhosis. Studies suggest that although simple fatty liver is a benign condition, nonalcoholic steatohepatitis may progress to fibrosis and lead to end-stage liver disease. It is strongly associated with obesity and insulin resistance and is currently considered by many as a hepatic component of the metabolic syndrome (Bugianesi *et al.*, 2005).

Nonalcoholic fatty liver disease is the most common cause of mild alteration of liver enzyme levels in the western world, and, according to the National Health and Nutritional Survey, point-prevalence is about 23 % among American adults (Harrison *et al.*, 2002). The biochemical picture includes mildly raised aminotransferase levels, and γ-glutamyl transferase (GGT) levels can be elevated up to 3 times the upper reference value in nearly half of patients in the absence of ethanol consumption (Brunt, 2004). The AST/ALT ratio greater than 1, which is observed in 61 % of patients with advanced fibrosis and 24 % of patients with no or initial fibrosis, is highly suggestive of advanced liver disease (Angulo *et al.*, 1999). Suspicion of nonalcoholic fatty liver disease is increased by the presence of conditions linked to the metabolic syndrome and insulin resistance (increased body mass index, diabetes, hyperlipemia, hypertension), although the disease may occur in patients without these associated factors (Brunt, 2004).

1.2.4. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the 5th most common tumor worldwide. The epidemiology of HCC exhibits two main patterns, one in North America (El-Serag, 1999) and Western Europe and another in non-Western countries, such as sub-Saharan Africa, central and Southeast Asia, and the Amazon basin, suggesting that both host and environmental factors are involved in its etiology. There is a two

to three fold higher incidence of carcinoma in females and is usually more common between the 3rd and 5th decades of life (Bosch, *et al.*, 1999; Kumar *et al.*, 2003). Hepatocellular carcinoma causes 662,000 deaths worldwide per year. Liver cells (hepatocytes) make up 80 % of the liver tissue, thus the majority of primary cancers arises from liver cells and is called hepatocellular carcinoma. Hepatocellular carcinoma, like any other cancer, develops when there is a mutation to the cellular machinery that causes the cell to replicate at a higher rate and/or results in the cell, avoiding apoptosis. (Dennis, 2007).

Once cirrhosis is developed, the rate of development of HCC is about 1.5 – 5 % per year in both hepatitis B virus (HBV) and hepatitis C virus (HCV) (Fattovich *et al.*, 1995; Fattovich *et al.*, 1997). The risk of HCC doubles in the case of those infected with both viruses (Fattovich, 1998). The mechanism of increased risk in HBV is thought to be related to the integration of HBV DNA into the host genome, which may block the activity of p^{53} (Feitelson *et al.*, 1993; Ueda *et al.*, 1995). The role of hepatitis B virus (HBV) infection in causing liver cancer is well established (Fattovich 1997; Fattovich 1995). Hepatitis C infection also associated with the development of liver cancer (El-Serag, 1999).

Cirrhosis caused by chronic alcohol consumption is the most common association of liver cancer in the developed world. When an individual with alcoholic cirrhosis has stopped drinking for ten years, develop liver cancer. It is unusual for an actively drinking alcoholic to develop liver cancer, because when drinking is stopped, the liver cells try to heal by the regeneration process. It is during this active regeneration that a cancer producing mutation can occur, which explains the occurrence of liver cancer after drinking has been stopped. Food infected with *Aspergillus flavus* (especially peanuts and corns stored during prolonged wet seasons) which produces aflatoxin, poses another risk factor for HCC. It is thought to cause liver cancer by producing changes (mutations) in the p^{53}

gene. These mutations work by interfering with the gene's important tumor suppressing functions (Puisieux *et al.*, 1991).

Female hormones (estrogens) and protein building drugs are associated with the development of hepatic adenoma that can later become malignant. Liver cancer will develop in up to 30 % of patients with hereditary hemochromatosis. Individuals with more types of cirrhosis of the liver are at an increased risk of liver cancer than are other causes.

It is a primary malignancy of the hepatocytes that generally leads to death within 6 months of the onset. HCC frequently arises in the setting of cirrhosis, appearing 20–50 years after the initial insult to the liver (Burrough *et al.*, 2004). The usual outcome is poor, because only 10–20 % of hepatocellular carcinoma can be removed completely by surgery. If the cancer cannot be completely removed, the disease is usually deadly within 3 to 6 months.

Symptoms of liver cancer include abdominal pain, unexplained weight loss or unexplained fever, ascites, jaundice, muscle wasting without any causative factors. It is often detected by ultrasound screening, and so can be discovered by health-care facilities much earlier than in developing regions such as Sub-Saharan Africa. Tumor markers of hepatocellular carcinoma include, α feto protein, des-γ carboxyprothrombin, α-L fucosydase and isoenzymes of γ glutamyl transferase.

Liver function tests are useful in detecting, diagnosing, evaluating severity, monitoring therapy and assessing the prognosis of the liver disease and liver function. The array of tests useful for these purposes include measurement in plasma of total bilirubin, albumin concentrations and the activity of enzymes such as the aminotransferases (AST and ALT), ALP, lactate dehydrogenase and GGT. By using a combination of these tests, it is possible to categorize broad types of liver disease, which can then be more accurately diagnosed through disease specific tests.

1.3. Bilirubin

Bilirubin consists of an open chain of four pyrrole-like rings (*tetrapyrrole*). In heme, by contrast, these four rings are connected into a larger ring, called a porphyrin ring. Bilirubin is created by the activity of biliverdin reductase on biliverdin. Bilirubin, when oxidized, reverts to become biliverdin once again. This cycle, in addition to the demonstration of the potent antioxidant activity of bilirubin, has led to the hypothesis that bilirubin's main physiologic role is as a cellular antioxidant (Baranano *et al.*, 2002).

Bilirubin is the product of hemoglobin catabolism within the reticuloendothelial system mainly by spleen, which is then transported to liver. Uptake of bilirubin across the hepatocyte sinusoidal membrane occurs by carrier mediated mechanism, which is shared by other organic anions. The uptake process is rapid, has a great capacity and is not rate limiting for hepatic bilirubin transport. Several carrier proteins that mediate bilirubin uptake into the hepatocytes have been identified. Once inside the hepatocytes, bilirubin appears to interact with membrane lipids such that it may transport from membrane to membrane (plasma membrane to endoplasmic reticular membrane). In endoplasmic reticulum, the COOH groups of one or both of its propionic acid side chains are esterified with glucuronic acid by the enzyme bilirubin UDP glucoronlyl transferase to form bilirubin mono or di glucuronides, which are more water soluble. The hepatocyte microtubular system, hepatic bile salt excretion, and membrane carrier proteins, all appear to facilitate the excretion of bilirubin glucuronides (conjugated bilirubin) into bile (Berk and Noyer, 1994).

In plasma, four bilirubin fractions are identified. 1) Unconjugated bilirubin (α-bilirubin), which is usually bound with albumin, 2) bilirubin mono glucuronate (β-bilirubin), 3) bilirubin di glucuronide (γ bilirubin) and 4) conjugated bilirubin bound to albumin. (δ-bilirubin or biliprotein) (Carl *et al.*, 2006).

Unconjugated bilirubin may increase because of augmented bilirubin production or decreased hepatic uptake or conjugation or both. In adults, the most common conditions associated with unconjugated hyperbilirubinemia are hemolysis and Gilbert's syndrome (Fevery and Blanckaert, 1986). Other, less frequent causes of unconjugated hyperbilirubinemia include reabsorption of large hematomas and ineffective erythropoiesis (Pratt and Kaplan, 2000).

In healthy people, conjugated bilirubin is virtually absent from serum mainly because of the rapid process of bile secretion (Green and Flamm, 2002). Bilirubin levels increase when the liver has lost at least half of its excretory capacity. Therefore, the presence of increased conjugated bilirubin is usually a sign of liver disease. Conjugated hyperbilirubinemia (usually <34 μmol/L) and concomitant, markedly elevated aminotransferase levels may suggest acute viral hepatitis or toxic or ischemic liver injury. Furthermore, this biochemical picture can be the presenting feature of autoimmune hepatitis (Krawitt, 1996; Alvarez *et al.*, 1999; Kessler *et al.*, 2004).

A purely cholestatic picture, with conjugated hyperbilirubinemia, an increase in ALP levels and a negligible increase in aminotransferase levels, may be present in cholestatic drug reactions (Lee, 2003; Velayudham and Farrell 2003). Sometimes, the same biochemical picture may be present in the late presentation of previously unrecognized autoimmune cholestatic diseases (primary biliary cirrhosis, primary sclerosing cholangitis). In these patients, the presence of other signs of chronic liver disease may facilitate diagnosis (Ponsioen and Tytgat, 1998; Morrison and Kowdley, 2000; Heathcote, 2000). Biliary obstruction can cause various degrees of conjugated hyperbilirubinemia. The severity of alteration depends upon the degree and duration of obstruction and the functional reserve of the liver. Biliary obstruction may have an abrupt onset and be preceded by typical symptoms

(right upper quadrant pain, nausea) or may be silent and progressive. With the presence or absence of concomitant aminotransferase alteration, a liver ultrasound is essential to identify and locate the obstacle to bile flow. Once the causal condition of conjugated hyperbilirubinemia has resolved, whatever be the cause, bilirubin serum levels decrease in a bimodal fashion. There is a first, rapid decrease and then a later, slower decrease caused by the binding of bilirubin to albumin and the formation of a complex (δ-bilirubin) that has the same half-life as serum albumin (Berk and Noyer, 1994; Fevery and Blankaert, 1986; Van Hootegem *et al.*, 1985).

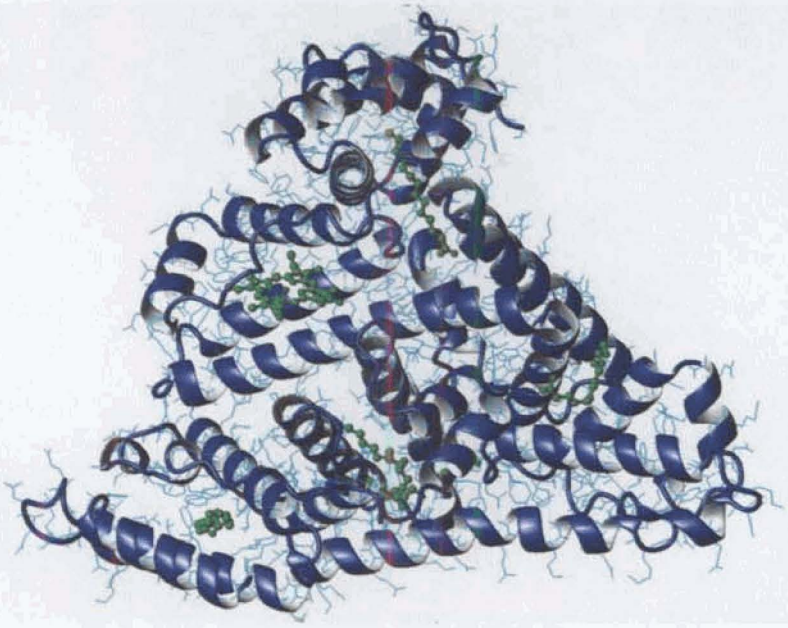
Gilbert's syndrome is a genetic disorder of bilirubin metabolism, which can result in mild jaundice, found in about 5 % of the population. Moderate rise in bilirubin may be caused by drugs (especially anti-psychotic, some sex hormones, and a wide range of other drugs), hepatitis (levels may be moderate or high), biliary stricture (benign or malignant). Very high levels of bilirubin may be caused by neonatal hyperbilirubinaemia, where the newborn's liver is not able to properly conjugate the bilirubin. Hyperbilirubinemia is also reported in large bile duct obstruction, e.g., stone in common bile duct, tumour obstructing common bile duct etc, severe liver failure with cirrhosis, severe hepatitis, Crigler-Najjar syndrome, Dubin-Johnson syndrome and Choledocholithiasis (chronic or acute).

1.4. Albumin

Albumin has a single polypeptide chain of 580 amino acids, with 17 intra chain SS bonds aligned in a mutiple loop structure. It has a molecular weight of 69 Kd and it contains 17 histidine residues (Vasudevan and Sreekumari, 2003). Albumin is one of only a few plasma proteins with no carbohydrate side chain. It is a very stable protein with a high net negative charge at physiological pH and partly as a result is very high solubility in water. There is one free SH group at position 34 that reacts completely with thiol compounds such as cysteine at physiological pH (Peters, 1996).

Albumin is primarily synthesized by the hepatic parenchymal cells except in early foetal life, when it is synthesized largely by the yolk sac. The synthetic rate is controlled primarily by colloidal osmotic pressure and secondarily by protein intake (Peters, 1996; Rothschild *et al.*, 1972). Liver produces about 12 grams of albumin per day and its half life is about 20 days (Vasudevan and Sreekumari, 2003). Catabolism occurs primarily by pinocytosis by all tissues. By the process of lysosomal catabolism proteins are converted to free amino acids for the synthesis of cellular proteins. The rate of pinocytosis is proportional to the local tissue metabolic rate.

The primary function of albumin is generally considered to be the maintenance of colloid osmotic pressure in both the vascular and extra vascular compartments. The presence of many charged surface groups and many specific binding sites, both ionic and hydrophobic gives albumin the ability to bind and transport a large number of compounds. This includes free fatty acids, phospholipids, cholesterol, metallic ions, amino acids, drugs, hormones and bilirubin. It functions as an amino acid source for pheripheral tissues and act as a buffer (Vasudevan and Sreekumari, 2003). The binding of albumin to endothelial membrane associated glycoproteins increases capillary permeability to small proteins that are important for metabolism in the extra vascular space. Albumin inhibits leukotriene and actin



production, thus reducing the inflammatory response of platelets and neutrophils. Albumin also possesses anti oxidant activity (Carl *et al.*, 2006).

Inflammatory disorders both acute and chronic are the most common causes of decreased plasma albumin levels. The most common cause of decreased plasma albumin levels are 1) hemodilution, 2) loss into the extra vascular space caused by increased vascular permeability, 3) increased consumption by cells locally and 4) decreased synthesis as a result of direct inhibition by cytokines (Ryffel *et al.*, 1994) 5) decreased release by hypokalaemia (Rothschild *et al.*, 1972). 6) small amounts are also lost into the gastrointestinal tract and the glomerular filtrate (Peters, 1996).

Plasma albumin measurements are useful in assessing the chronicity and severity of liver diseases. Low serum albumin, often found with severe chronic liver disease is probably due to its reduced synthesis. The plasma albumin level is an indication of the synthetic capacity of liver.

1.5. Liver enzymes

There are four liver enzymes that are commonly used in the diagnosis of liver diseases. They are aspartate aminotransferase (AsT; EC:2.6.1.1), alanine aminotransferase (AlT; EC 2.6.1.2), alkaline phosphatase (ALP; EC 3.1.3.1) and γ-glutamyltransferase (GGT; EC 2.3.2.2). AlT and GGT are present in several tissues, but plasma activities primarily reflect liver injury. AsT is found in liver, muscle and to a limited extent in red blood cells. Bone and liver are good sources of ALP in normal individuals, though it is seen in a number of other tissues. Based on tissue distribution, AlT and GGT would seem to be the most specific markers for liver injury (Carl *et al.*, 2006).

1.5.1. Aminotransferases

Injury to liver, whether acute or chronic, eventually results in an increase in serum concentrations of aminotransferases. AST and ALT are enzymes that catalyze the transfer of α-amino groups from aspartate and alanine to the α-keto group of ketoglutaric acid to generate oxaloacetic and pyruvic acids respectively, which are important contributors to the citric acid cycle. Both enzymes require pyridoxal- 5'-phosphate (vitamin B6) in order to carry out this reaction, although the effect of pyridoxal-5'-phosphate deficiency is greater on ALT activity than on that of AST (Dufour *et al.*, 2000; Vanderlinde, 1986). This has clinical relevance in patients with alcoholic liver disease, in whom pyridoxal-5'-phosphate deficiency may decrease ALT serum activity and contribute to the increase in the AST/ALT ratio that is observed in these patients (Cohen and Kaplan, 1979; Diehl *et al*, 1984). Both aminotransferases are highly concentrated in the liver. AST is also diffusely represented in the heart, skeletal muscle, kidneys, brain and red blood cells, and ALT has low concentrations in skeletal muscle and kidney (Panteghini, 1990). An increase in ALT serum levels is, therefore, more specific for liver damage. In the liver, ALT is localized solely in the cellular cytoplasm, whereas AST is both cytosolic (20 % of total activity) and mitochondrial (80 % of total activity) (Rej, 1989). Zone 3 of the hepatic acinus has a higher concentration of AST, and damage to this zone, whether ischemic or toxic, may result in greater alteration to AST levels. Aminotransferase clearance is carried out within the liver by sinusoidal cells (Kamimoto *et al*, 1985). The half-life in the circulation is about 47 hours for ALT, about 17 hours for total AST and, on average, 87 hours for mitochondrial AST (Dufour *et al.*, 2000).

Patients with a marked increase in aminotransferase levels more than 10 times the upper reference limit, typically have acute hepatic injury. However, data from a series of patients with acute hepatic injury due to viral hepatitis suggest that the most sensitive and specific aminotransferase threshold level to identify acute

injury lies within the moderate range of increase of 5–10 times the upper reference limit, at 200 IU/L for AST and 300 IU/L for ALT (Rozen *et al.*, 1970). Thus, the academic attribution of cause and "severity" of acute damage on the basis of the magnitude of enzyme elevation may sometimes be misleading, since there can be grey areas in which a range of causes overlap. Moreover, the degree of elevation varies during the course of injury and depends on the time of enzyme levels tested. Despite these ambiguities, the magnitude and rate of change of aminotransferase alteration may provide initial insight into a differential diagnosis.

Very high aminotransferase levels of more than 75 times the upper reference limit indicate ischemic or toxic liver injury in more than 90 % of cases of acute hepatic injury, whereas they are less commonly observed with acute viral hepatitis (Dufour *et al.*, 2000). In ischemic or toxic liver injury, AST levels usually peak before those of ALT because of the enzyme's peculiar intralobular distribution (Dufour and Teot, 1988; Singer *et al.*, 1995; Seeto *et al.*, 2000). Zone 3 of the acinus is more vulnerable to both hypoxic (hepatocytes are exposed to an already oxygen-poor milieu) and toxic (hepatocytes are richer in microsomal enzymes) damage. Furthermore, in ischemic injury aminotransferase levels tend to decrease rapidly after peaking. In about 80 % of patients with ischemic injury, the serum bilirubin level is lower than 34 μmol/L. It is important to stress that a decrease in aminotransferase levels alone after a marked increase does not have prognostic meaning, since both resolution and massive hepatic necrosis may draw a similar biochemical picture. In this setting, patients with high bilirubin serum levels and prolonged prothrombin time should be closely monitored for the risk of hepatic failure. In cases of acute viral hepatitis, aminotransferase levels usually peak before jaundice appears and have a more gradual decrease thereafter, and there is a greater increase in serum bilirubin levels (Clermont and Chalmers, 1967). Jaundice occurs in about 70 % of cases of acute hepatitis A infection, 33 – 50 % of cases of acute

hepatitis B infection and 20 – 33 % of cases of acute hepatitis C infection (Dufour *et al.*, 2000).

1.5.2. Alkaline phosphatase

Alkaline phosphatases are zinc metalloenzymes that release inorganic phosphate from several organic orthophosphates. They are present in nearly all tissues. Their natural substrates may include pyrophosphate, phosphoserine and phosphoethanolamine. Intestinal ALP acts as a calcium dependent ATPase. In liver, alkaline phosphatase can be found histochemically in the microvilli of the bile canaliculus and on the sinusoidal surface of hepatocytes. Alkaline phosphatase exists in tissue specific isoforms, some of which are true isoenzymes in that they are the products of separate genes. Biliary, liver, bone, placental, renal and intestinal isozymes are identified (Rosalki, 1975; Nemesanszky, 1986). The alkaline phosphatases from liver, bone and kidney are thought to be coded for the same gene, while the ALP from intestine and placenta has different genes. Alkaline phosphatase activity in normal serum is mainly due to the isoforms from bone and liver with near equal proportions. Intestinal ALP may also contribute up to 20 % of total activity. Placental enzyme may appear in mid-pregnancy.

ALP activity varies with age and sex and with several other factors. There are two peaks, one in neonatal period and the other in adolescence. Levels also tend to rise in older subjects with increased liver ALP in elderly men and bone ALP in elderly women (Kuwana *et al.*, 1988). Cellulose acetate electrophoresis identifies two liver fractions, a major fraction of α-1 globulin mobility accompanied by a fraction of α-2 globulin mobility, together with bone isoenzymes. Intestinal isoenzyme is easily identified by its β globulin mobility. Other methods for the detection of ALP isoenzymes are by using monoclonal antibodies and wheat germ lectin precipitation (Rosalki and Foo, 1984).

The serum ALP activity rises in many liver diseases, the highest level occurring in intrahepatic or extrahepatic obstruction to the flow of bile. In acute viral hepatitis ALP is usually either normal or moderately raised but up to 40 % of patients have levels two and half times the upper reference limit. The serum alkaline phosphatase is increased by drugs that cause cholestasis liver disease. Elevation was also reportedly caused by cimetidine (Payne *et al.*, 1982), frusemide (Math, 1982), phenobarbitone (Balazs *et al.*, 1978) and phenitoin (Moss, 1975). Low ALP has been found in Wilson's disease presenting with hemolytic anemia and evidence of severe liver dysfunction (Shaver *et al.*, 1986).

Liver and bone diseases are the most common causes of pathological elevation of ALP levels, although ALP may originate from other tissue, such as the placenta, kidneys or intestines or from leukocytes (Fishman, 1990). The third trimester of pregnancy (placenta origin) and adolescence (bone origin) are associated with an isolated increase in serum ALP levels (Dufour *et al.*, 2000). Hepatic ALP is present on the surface of bile duct epithelia. Cholestasis enhances the synthesis and release of ALP, and accumulating bile salts increase its release from the cell surface (Schlaeger *et al.*, 1982; Moss, 1997). ALP half-life in the circulation is about 1 week (Dufour *et al.*, 2000). These characteristics explain why ALP levels usually rise late in bile duct obstruction and decrease slowly after resolution.

In some patients (e.g., pregnant women, adolescents) the reason for increased ALP levels may be straight forward, but in other patients it is necessary to identify the origin of the enzyme. This task can be accomplished in 2 ways: assessment of GGT levels or dosage of ALP isoenzymes. From a practical point of view, measurement of GGT is preferred since it relies on automated analysis rather than on more sophisticated and expensive techniques. The degree and rate of enzyme alteration may provide minor and nonspecific clues to diagnosis, but the presence of

symptoms and the patient's history, with particular emphasis on comorbid conditions, may provide fundamental clues. Liver ultrasound may reveal the presence of bile duct dilation, demonstrate signs of chronic liver disease or even liver cirrhosis, and identify hepatic masses.

Drug-induced liver injury may present with a cholestatic pattern (preferential increase in ALP or ALT/ALP ratio < 2), although the degree of ALP alteration is variable and may be accompanied by hyperbilirubinemia (Velayudham and Farrell, 2003). Commonly used drugs such as antihypertensives (e.g., angiotensin converting enzyme inhibitors) or hormones (e.g., estrogen) may cause cholestasis and can be overlooked. It has been found that ALP acts as an acute phase reactant in Hodgkin`s disease, congestive heart failure and in infectious and inflammatory diseases (Brensilver and Kaplan, 1975; Parker *et al.*, 1989) Normal alkaline phosphatase is also observed in patients with primary biliary cirrhosis (Sherlock and Scheuer, 1973) and in primary sclerosing cholangitis (Cooper and Brand, 1988).

Increased serum intestinal ALP activity is found in cirrhosis and may result from diminished hepatic uptake, perhaps due to disruption of receptors for intestinal alkaline phosphatase of the liver cell surface, or due to diminished hepatic excretion of catabolism (David and Vincent, 1998). Tumors may secrete ALP into plasma. Some tumors may produce specific isoenzymes- for example Regan, Nagao and Kasahara isoenzymes may be found in patients with carcinoma of the bile duct. The Kasahara isoenzyme (a fetal intestinal like phosphatase) has been found in the serum of about 30 % of patients with primary liver cell carcinoma (Higashino *et al.*, 1975).

1.5.3. Gamma glutamyl transferase

Gamma glutamyltransferase (GGT) or gamma glutamyltranspeptidase (GGTP) is a membrane bound glycoprotein that catalyses the transfer of γ glutamyl

groups from γ-glutamyl peptides particularly glutathione to other peptides to amino acids and to water. It is found mainly in the membranes of cells with a high rate of secretory or absorptive activity. Large amounts are present in the kidneys, pancreas, liver, intestine and prostate and is also found in many other tissues. The γ-GT activity in bile is approximately 100 times greater than in normal serum (Rosalki, 1975). Several isoforms of GGT have been described but there is no clear evidence of tissue specificity. The heterogeneity is related to the number of sialic acid residues, to the degree of glycosylation and to binding to lipoproteins (Rosalki, 1984). The half life of GGT has been reported as 1-4 days. It is proposed that the enzyme is removed by receptor - mediated endocytosis by liver macrophages.

In liver, GGT is present in hepatocytes and biliary epithelial cells, renal tubules, and the pancreas and intestine. The mechanisms of alteration are similar to those described for alkaline phosphatase. It is a microsomal enzyme, and its activity can be induced by several drugs, such as anticonvulsants and oral contraceptives (Rosalki, 1971). Elevated GGT levels can be observed in a variety of nonhepatic diseases, including chronic obstructive pulmonary disease and renal failure, and may be present for weeks after acute myocardial infarction. Increased serum levels observed in alcoholic liver disease can be the result of enzyme induction and decreased clearance. In these patients, GGT serum levels can be markedly altered (> 10 times the upper reference value), whereas ALP levels may be normal or only slightly altered (GGT/ALP ratio > 2.5). The whole spectrum of liver diseases, regardless of cause, may be responsible for altered GGT serum levels. In particular, GGT levels may be 2–3 times greater than the upper reference value in more than 50 % of the patients with nonalcoholic fatty liver disease and above the upper reference value in about 30 % of patients with chronic hepatitis C infection (McCullough *et al.*, 2002; Giannini, *et al.*, 2001). Furthermore, an increase in GGT levels in patients with chronic liver disease is associated with bile duct damage and fibrosis (Giannini *et al.*, 2001). Thus, because of its lack of specificity but high sensitivity for liver

disease, GGT can be useful for identifying causes of altered ALP levels, or elevated levels, together with other biochemical abnormalities (AST/ALT ratio > 2) may support the diagnosis of alcoholic liver disease (Cohen and Kaplan, 1979).

1.6. Acute phase proteins

Acute phase proteins (APP) or acute phase reactants (APR) is a generic name given to a group of approximately 30 different biochemically and functionally unrelated proteins. These proteins are secreted by hepatocytes (Ruminy *et al.*, 2001) and their levels in the serum are either increased (positive acute phase reactants) or reduced (negative acute phase reactants) approximately 90 minutes after the onset of a systemic inflammatory reaction. Acute phase proteins are synthesized predominantly in the liver with all hepatocytes possessing the capacity to produce the entire spectrum of these proteins in response to tissue injury as a result of neoplasia, trauma and infection (Heinrich *et al.*, 1990). Following stimulation of single hepatocyte within individual lobule, one observes a stimulation of further hepatocytes and this process continues until almost all hepatocytes produce these proteins and release them into the circulation. The various APP differ markedly in the rise or decline of their plasma levels and also in their final concentrations. APP generates a characteristic serum protein profile. Levels of elevated expression can differ widely from species to species and some proteins that can function as APP in one species may not be an acute phase protein in another species (Baumann, 1988).

Acute phase proteins regulate immune responses, function as mediators and inhibitors of inflammation, act as transport proteins for products generated during the inflammatory process, and play an active role in tissue repair and tissue remodelling. Acute phase response is a protective physiological reaction of the organism to disturbances of its homeostasis due to inflammation caused by tissue injury, infection or neoplastic growth (Heinrich *et al.*, 1990; Fey and Gauldie, 1990; Baumann and Gauldie, 1994). Van Molle *et al* (1999) have suggested that at least

some APP might constitute an inducible system of factors protecting against cell death by apoptosis. They have observed that α-1 antitrypsin and α-1 acid glycoprotein activate the major executioners of apoptosis, caspase 3 and caspase 7.

Some of the APP behaves like cytokines, C-reactive protein, for example activates macrophages, some influence the chemotactic behaviour of cells, some possess antiproteolytic activity and presumably block the migration of cells onto the lumen of blood vessels thus helping to prevent the establishment of generalized systemic inflammation. A failure to control these processes or an uncontrolled APR eventually has severe pathologic consequences.

The elevated serum concentrations of certain acute phase proteins are of diagnostic relevance and also of prognostic value. Their measurement, for example, allows inflammatory processes to be distinguished from functional disturbances with similar or identical clinical pictures. Under normal circumstances an APR is not observed with functional disturbances that are not the result of an inflammatory process, thereby allowing the differentiation between failure of function and organic disease (Dofferhoff *et al.*, 1992).

Some acute phase reactions are observed in chronic disorders such as rheumatoid arthritis and chronic infections, while malignant diseases are almost invariably associated with an APR and therefore the determination of acute phase protein cannot be used for differential diagnosis in these instances. There are many diseases in which the rise in the synthesis of acute phase proteins parallels the degree and progression of the inflammatory processes (Ramadori *et al.*, 1999).

Acute phase response is a systemic reaction of the organism to non specific systemic stimuli that is accompanied by increased production of a cytokine cascade, which includes tumor necrosis factor-α (TNF-α), IL-1, IL–6, IL–11, leukemia

inhibitory factor and oncostatin M (Heinrich *et al.*, 1990; Fey and Gauldie, 1990; Baumann and Gauldie, 1994). Characteristic of AP response after a local injury includes the release of cytokines, which in turn induce a systemic reaction manifested by fever elevated secretion of glucocorticoids and changes in the concentration of acute phase proteins. These APP are either upregulated (positive APP) or down regulated (negative APP) during the acute phase response. Protease inhibitors, blood coagulation factors, transport proteins and compliment components are examples for positive APP, which are commonly upregulated 2–10 fold on both the mRNA and protein levels. Typical negative APP include albumin and transferrin. The spectrum of acute phase proteins produced in hepatoma cells however varies quantitatively and qualitatively between the different cytokines studied (Semenza and Wang, 1992, 1993).

The two cytokine mediators IL-1 and IL-6 have been used to classify APP into two subgroups. Type 1 APP are those that require the synergistic action of IL-6 and IL-1 for maximum synthesis e.g., CRP, serum amyloid A and α-1 acid glycoprotein. Type 2 APP are those that require IL-6 only for maximum induction e.g., fibrinogen chains, haptoglobin, α-2 macroglobulin. Expression of genes encoding type 2 APP is suppressed rather than being enhanced frequently by IL-1 (Ramadori *et al* 1999; Fey *et al*, 1994). Additive synergistic, co operative and antagonistic effects between cytokines and other mediator substances influencing the expression of APP do occur and have been observed in almost all combinations. Many cytokines also show differential effects, inducing the synthesis of one or two APP but not others (Benigni *et al.*, 1996).

Several animal models were used for the analysis of the acute phase response including rats (Baumann *et al.*, 1983) mice (Beaudet *et al.*, 1982; Baumann *et al.*, 1984) and rabbits (MacIntyre *et al.*, 1983), each of which has a slightly different subset of proteins that responds to the appropriate stimulation *in vivo*. *in vivo*

studies are complicated by the multiplicity of cell types in the body and pose some questions whether the stimulus acts directly or indirectly upon the hepatocytes. Primary hepatocyte cultures have shown that stimulating agent such as turpentine and bacteria do not operate directly on hepatocytes. Rupp and Fuller (1979) have shown that one acute phase protein fibrinogen was elevated when supernatants derived from isolated peripheral monocytes were added to primary rat hepatocytes *in vitro*. This demonstration clarified the role of cellular intermediates in the acute phase response although the primary hepatocytes culture system is not a totally pure population of parenchymal cells. Sipe *et al.*, (1982) have reported the induction of serum amyloid A after the injection of mice with purified fractions of mouse IL-1 and endogenous pyrogens. Woloski and Fuller (1985) have shown that a separate factor, which they have called hepatocytes stimulating factor elevated fibrinogen in primary rat hepatocytes cultures.

The altered hepatic transcription of the APP genes represents an adaptive response to minimize damage during the acute phase response. Activated macrophages invade damaged tissues and release a number of factors into the blood stream including interleukin-1β. This 17.4 Kd lymphokine reproduces most acute phase changes when administered to rats. Some of these *in vivo* responses are also reproduced by the administration of recombinant IL-1β to hepatoma cells grown *in vitro* (Karin *et al.*, 1985).

There are many diseases in which the rise in the synthesis of acute phase proteins parallels the degree and progression of the inflammatory processes. The co-ordinated expression of many acute phase proteins as a direct consequence of the activities of several cytokines can be explained, at least in part, by the fact that the regulatory sequences of the genes encoding these acute phase proteins contain so-called cytokine response elements. These elements are recognized specifically by

transcription factors that mediate the activity of these genes in a cell- and/or tissue-specific manner (Richards *et al.*, 1991).

Interleukin-1 and also interferon-γ reduce some of the effects of IL-6. Some of the effects of IL-2 and IL-6 are antagonized by TGF-β. The combined action of two or even more cytokines may produce effects that no factor on its own would be able to achieve. In cultured HepG2 hepatoma cells IL-1, IL-6, TNF-α and TGF-β induce the synthesis of antichymotrypsin and at the same time repress the synthesis of albumin and α-fetoprotein (AFP). The synthesis of fibrinogen is induced by IL-6 and this effect is, in turn, suppressed by IL-1α, TNF-α or TGF-1β. The increased synthesis of Haptoglobin mediated by IL-6 is suppressed by TNF-α. Insulin inhibits the synthesis of some negative acute phase proteins (prealbumin, Transferrin, and fibrinogen, in HepG2 hepatoma cells (Ikawa and Shozen, 1990).

Different patterns of cytokines are involved in systemic and localized tissue damage, which is supported by observations with knock-out mice for IL-1 and IL-6. Inflammatory acute phase response after tissue damage or infection is severely compromised in IL-6 knock-out mice, but only moderately affected after challenge with bacterial lipopolysaccharides (Kopf *et al*, 1994). Fattori *et al* (1994) show that in the absence of IL - 6, the induction of acute phase proteins is dramatically reduced in response to turpentine injections but that parameters are altered to the same extent both in wild-type and IL - 6 deficient mice following injection of bacterial lipopolysaccharides. These mice, however, produce three times more TNF-α than wild-type controls. Bopst *et al* (1998) observed a normal acute phase reaction to both turpentine and bacterial lipopolysaccharides in TNF-β knock-out mice. They have reported a striking absence of elevated major acute phase proteins, serum amyloid P and serum amyloid A, in mice deficient in TNF-β and IL-6. Fantuzzi *et al* (1996) demonstrated that IL-1β knock-out mice, on the other hand, show a normal response to bacterial lipopolysaccharides.

1.6.1. C-reactive protein

The major site of synthesis of C-reactive protein is the hepatocyte (Hurlimann *et al.*, 1966, Kushner and Feldmann, 1978). CRP is secreted from the liver cells and can be observed in the serum as an annular disc consisting of five identical mature polypeptide subunits (Osmond *et al.*, 1977). The genomic sequence of human CRP indicates that the precursor subunit is composed of 206 amino acids of mature peptide as well as an 18 amino acid signal sequence assay (Lei *et al.*, 1985).

C-reactive protein is named for its capacity to precipitate the somatic C polysaccharide of *Streptococcus pneumoniae,* was the first acute phase protein to be described and is an exquisitely sensitive systemic marker of inflammation and tissue damage (Pepys *et al.*, 2003). CRP has been shown to bind specifically to phosphorylcholine in a calcium-dependent manner (Volanakis and Kaplan, 1971). Another ligand with higher affinity than phosphorylcholine, chromatin, has been shown to bind to CRP (Robey *et al.*, 1984). The binding of these ligands to CRP has been shown to initiate the classical complement pathway by the subsequent binding of complement component Clq (Kaplan and Volanakis, 1974, Robey *et al.*, 1984). In addition, CRP has been found to play an important role in the platelet-dependent killing of immature schistosomes (Bout *et al.*, 1986). CRP also has been shown to bind to a specific class of T-lymphocytes (James *et al.*, 1983) large granular lymphocytes. CRP, the major acute phase reactants in humans, derives mainly from hepatocytes in response to IL - 6 and is then secreted into the systemic circulation (Ross, 1999). CRP down regulates endothelial nitric oxide synthase resulting in decreased release of NO and this facilitates endothelial cell apoptosis and inhibition of angiogenesis (Libby, 2002).

Of the markers of inflammation, CRP has been shown in multiple prospective studies to predict the incidence of recent myocardial infarction, stroke,

peripheral vascular disease and sudden cardiac death (Ridker, 2001, Ridker *et al* 2003). Recent studies have shown that elevated CRP is strongly associated with various characteristics of the metabolic syndrome (Yudkin *et al.*, 1999; Festa *et al.*, 2000; Ridker *et al* 2003).

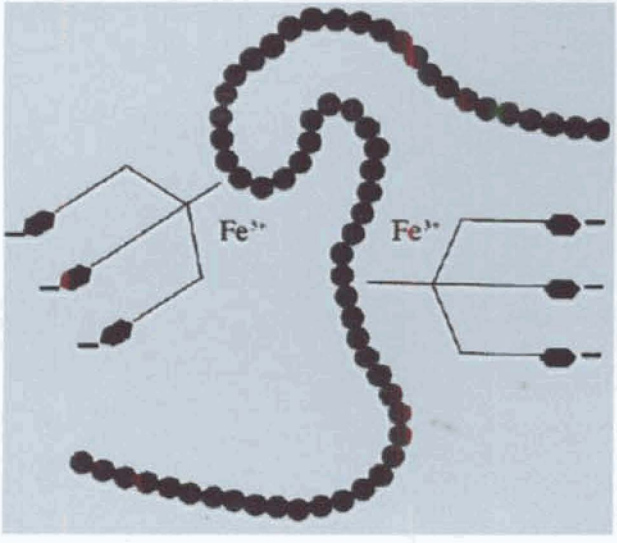
The levels of CRP increased in parallel with the progression of chronic liver diseases. Levels of α-2 macroglobulin is not changed with chronic hepatitis or liver cirrhosis, while those in patients with hepatocellular carcinoma are significantly higher than in controls or liver cirrhosis. Serum hepatocyte growth factor (HGF) showed a positive correlation with CRP and a negative correlation with albumin. The serum levels of acute phase proteins such as albumin and α-2 macroglobulin are more closely associated with the degree of hepatic dysfunction than serum HGF levels (Shiota *et al.*, 1995). Highly enhanced fucosylation of serum glycoproteins was found in HCC compared with liver cirrhosis and that the combination of measurements of fucosylated alpha feto protein or transferrin was useful for the diagnosis of HCC (Naitoh *et al.*, 1999). Serum CRP is not a good marker for HCC, however very high values of CRP in patients with cirrhosis may suggest the presence of a diffused type HCC (Lin *et al.*, 2000).

C-reactive protein is the prototype acute phase protein in humans (Mortensen, 2001) and its synthesis is stimulated by cytokines, especially IL-1β and IL–6. Transcription factors involved in IL-6 mediated CRP synthesis included signal transducer and activator of transcription 3 and members of the C/EBP family, especially C/EBP α, β and δ (Ghosh and Karin, 2002; Moscat *et al.*, 2003).

CRP synthesis increases during infections, allergic complications of infections, inflammatory disease, necrosis, trauma and malignancy (Pepys and Hirschfiel, 2003). The elevation of serum CRP is known in various liver diseases (Atono *et al.*, 1989; Lee *et al.*, 1989; Murakami *et al.*, 1989). The CRP

concentration is a useful nonspecific biochemical marker of inflammation, measurement of which contributes importantly to a) screening for organic disease, b) monitoring of the response to treatment of inflammation and infection and c) detection of inter current infection in immuno compromised individuals, and in the few specific diseases characterized by the modest or absent acute phase responses (Pepys *et al.*, 2003).

1.6.2. Transferrin



The iron binding protein of the plasma is transferrin, which is very similar to lactoferrin found in granulocytes and milk. It is a monomeric glycoprotein with 79.6 Kd and made up of 679 amino acids organized in 2 homologous domains, each containing an iron binding site (van Eden and Young, 1995). It is synthesized almost exclusively in the liver, with lesser amounts in the choroids plexus of the brain. Plasma levels are regulated primarily by the availability of iron. In iron deficiency, plasma transferrin level rise and on successful treatment with iron return to normal. It has a half life of approximately 8 -10 days.

Transferrin is involved in iron metabolism. Apotransferrin binds with iron absorbed from the intestine or released from catabolism of hemoglobin to form transferrin. The iron is then transported to storage sites such as the liver and the endothelial system and to sites of synthesis of iron containing compounds especially the erythropoietic tissue. These cells have surface receptors for transferrin (Lash and Saleem, 1995). After binding, transferrin iron complex is internalized into

caltherin coated endosome that lowers the internal pH, resulting in the release of iron from transferrin. Iron is transported across the vesicle membrane for utilization or storage within the cell and the transferrin receptor complex recycles back to the cell surface, where apotransferrin is released at the higher pH of blood (Aisen *et al.*, 2001).

High levels of transferrin are seen in pregnancy and during estrogen administration. Transferrin is a negative acute phase protein, and low levels are observed in inflammation, malignancy, liver disease, malnutrition, protein losing enteropathies (Carl *et al.*, 2006).

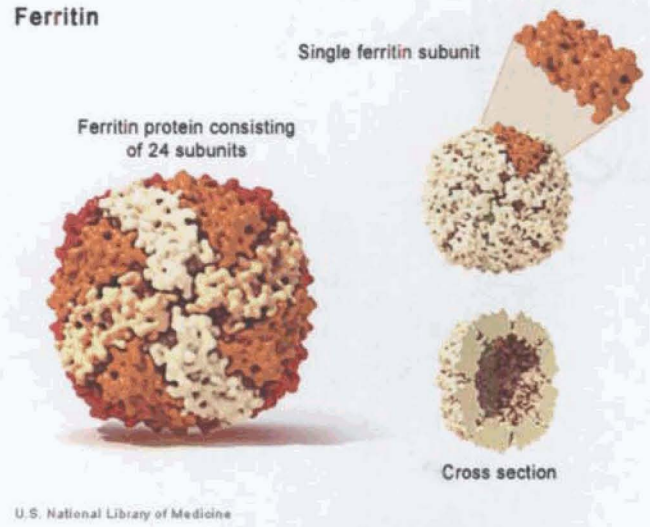
The amino acid back bone of transferrin contains two side chains at Asn 413 and Asn 611, which may bear bi or tri antinnary oligosaccharide side chains with terminal sialic acid groups. In a healthy patient the majority of the blood transferrin molecules carry 4 or 5 sialic acid groups. However, alcoholic patients possess transferrin with either no or less number of sialic acid groups. Such sialic acid group deficient transferrin level is found to be increased in alcoholic liver cirrhotic patients (Stibler, 1991). Serum acute phase proteins exist in different glycoforms. Abnormally glycosylated transferrin and other glycoproteins are found to be increased in different types of diseases including liver diseases. Besides transferrin, other clinically relevant proteins exist in differently glycosylated isoforms including glycosylated markers for cancers and other diseases.

Insulin is able to inhibit the synthesis of prealbumin, transferrin and fibrinogen (Thompson *et al.*, 1991). The micro heterogeneity analysis of human serum transferrin is useful for distinguishing patients with hepatocellular carcinoma from those with liver cirrhosis and normal controls (Suzuki *et al.*, 1996). Both albumin and transferrin gene expression has been shown to be regulated by various

agents such as cell density, human growth hormone and temperature (Sporn and Roberts, 1983).

1.6.3. Ferritin

Ferritin is a ubiquitous iron storage protein, the shell of which consists of a mixture of 24 heavy (H-21,000 Da) and light (L-19,000 Da) subunits (Theil, 1987). The subunits are roughly cylindrical in shape and form nearly a spherical shell that encloses a central core containing upto 4500 atoms of iron in the form of ferric hydroxyphosphate. *in vitro*, ferritin behaves as iron storage protein. Human ferritins are made up of two types of subunits (H and L subunits) in varying proportions. The gene for the L subunit is located on chromosome 19 and the gene for the H subunit is found in chromosome 11 (Worwood, 1990). Apoferritin will bind and oxidise Fe^{2+} and deposit Fe^{3+} within the protein. The release of iron may be effected by reducing agents.



Studies in animal cell culture show that apoferritin is synthesized in response to iron administration and this control is largely exercised at the level of translation (Worwood, 1990). The 5' end of untranslated ferritin mRNA contains 28 base sequence that forms a stem loop structure. This has been termed an iron response element. A cytoplasmic protein that binds to this sequence and prevents translation has been also been identified. In the presence of iron, this protein is unable to bind to the mRNA, and polysomes forms to proceed translation.

Detailed investigation of L-subunit mRNA expression revealed that translational control mechanisms regulate L-sub unit synthesis in response to IL-1β in human hepatoma cells. Northern blot and RNase protection analyses show that L-ferritin mRNA levels in HepG2 cells are unaffected by IL-1β treatment. The response of ferritin synthesis to IL–1β is accompanied by a redistribution of L-ferritin mRNA towards the polyribosomes consistent with an increase in translational efficiency. This occurs within two hours of cytokine administration and persists for at least 14 hours. Rat liver and spleen ferritin synthesis is elevated 3-4 fold 6 hours after the onset of an experimentally induced inflammatory response (Konijn and Hershko, 1977; Campbell *et al.*, 1989). Konijn *et al.*, (1981) suggested that increased ferritin synthesis occurs as the result of translational mechanisms since cytoplasmic extracts taken from rat liver reproduced this induction in the absence of nuclei *in vitro*. L-subunit mRNA was shown to be recruited from mRNAs to polyribosomes in rat liver and spleen cells 12 h after a turpentine induced inflammation (Campbell *et al.*, 1989). The mRNAs for both H and L-ferritins are translationally activated within the first 2 hours of administering iron to human and rat hepatoma cells (Rogers and Munro, 1987), human erythroleukemia cells (K562) (Rouault *et al.*, 1988) and mouse fibroblast cell lines (Walden and Tahch, 1986). In intact animals, a similar induction by iron results in a 10–20 fold increase in liver ferritin synthesis (White and Munro, 1988).

1.6.4. Ceruloplasmin

Ceruloplasmin is a major plasma protein containing most of the circulating copper (Ryden and Bjork, 1976; Linder and Hazegh-Azam,1996). Ceruloplasmin is an α2-glycoprotein mainly synthesized in the liver parenchymal cells with small amounts synthesized by macrophages and lymphocytes and secreted into the serum as the major copper transporting protein (Sas Kortsak and Bearn, 1965). This glycosylated serum protein has a molecular weight of 132 Kd and contains 6-8 copper molecules. Copper appears to be essential for the normal folding of the

polypeptide chain and possibly for normal CHO side chain attachment. The peptide chain is formed first and copper is added from an intra cellular ATPase. Apo Cp is synthesized even in the absence of copper or ATPase. In this condition most of it is degraded intracellularly but moderate amounts are released into the circulation, where Apo Cp has a very short half life.

Numerous functions have been attributed to ceruloplasmin, including a crucial role in iron metabolism through its peroxidase activity (Young *et al.*, 1997; Mukhopadhya *et al.*, 1998). It exhibits oxidase activity and oxidizes among other substrates, Fe^{2+} to Fe^{3+} that can then be stored by ferritin and transported into cells by transferrin (Ozaki and Johnson,1969; Harris *et al.*, 1999). It is involved in the antioxidant functions in the prevention of the formation of free radicals in serum (Gutteridge, 1983; Miyajima *et al.*, 1996; Richardson *et al.*, 1999). It is also involved in a number of metabolic processes related to copper metabolism (Harris, 1993), biogenic amines and nitric oxide metabolism (Bianchini *et al.*, 1999). A lack of ceruloplasmin leads to iron accumulation in the liver and finally to liver damage (Harris *et al.*, 1999; 1995).

Ceruloplasmin is a member of the acute phase protein family, and, consequently, its serum level is increased during inflammation as well as in various malignancies (Gitlin, 1988; Ramadori *et al.*, 1988). Ceruloplasmin is absent in aceruloplasminemia (Loreal *et al.*, 2002) and may be slightly decreased in other diseases such as fulminant hepatitis (Walshe *et al.*, 1962) and genetic hemochromatosis (Laine *et al.*, 1992). Serum ceruloplasmin is found to be decreased in decompensated cirrhosis with hepatic failure (Cauza *et al.*, 1997).

Dominique *et al.*, (2001) reported an increase in the ceruloplasmin level to extremely high levels during the development of HCC in ATIII–Tag transgenic mice and investigated the factors responsible for this increased synthesis.

Ceruloplasmin is found in the globulin fraction in the mammalian plasma. It shows significant size and charge heterogeneity because of differences in glycosylation, the number of copper atoms present, peptide chain variations, secondary to alternative DNA splicing and polymerization. In addition, it is very susceptible to proteolysis both *in vivo* and *in vitro*, by many proteases including trypsin, plasmin, leucocyte elastase and a plasma metalloproteinase. There are two important molecular isoforms, one predominating in bile (125 Kd) and the other in plasma (132 Kd). The biliary form is important for copper excretion, which is absent in Wilson's disease.

Serum ceruloplasmin is an important diagnostic marker in Wilson's disease, in which the plasma level is usually reduced. Low ceruloplasmin is also seen in neonates, Menke's disease, Kwashiorkar and marasmus, protein losing enteropathy, nephrotic syndrome, severe hepatic insufficiency, copper deficiency and in hereditary hypoceruloplasminaemia (David and Vincent, 1994).

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AIM OF THE STUDY

1) To determine the amount of bilirubin, albumin, serum enzymes (Aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase) and acute phase proteins (C-reactive protein, transferrin, ferritin, ceruloplasmin) in the serum of liver disease patients like nonalcoholic liver cirrhosis (NALC), alcoholic liver cirrhosis (ALC), hepatocellular carcinoma (HCC).

2) To compare the above serum parameters between control and all the diseased categories (NALC, ALC and HCC categories) of this study.

3) To compare the above serum parameters among the diseased categories (NALC, ALC and HCC categories).

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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 werefemales. 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 transferse) and serum acute phase proteins (C–reactive proteins, transferrin, ferritin and ceruloplasmin).

All the pareameters 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 sulphanylic 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:

Contents added	**Blank**	**Standard**	**Test**
Working reagent	500 µl	500 µl	500 µl
Distilled water	25 µl	-	-
Standard	-	25 µl	-
Test	-	-	25 µl

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:

Total bilirubin mg/dl = $\frac{\text{OD of test}}{\text{OD of standard}}$ X 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:

Contents added	**Blank**	**Standard**	**Test**
Albumin reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	-	-
Standard	-	10 µl	-
Test	-	-	10 µl

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.

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \longrightarrow \text{oxaloacetate} + \text{glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \longrightarrow \text{Malate} + NAD^+$$

Composition:

Reagent A: Tris 121 mMol/L, Laspartate 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:

$$\text{The AST concentration of the sample} = \Delta A/\text{Min} \times \frac{Vt \times 10^6}{\varepsilon \times l \times Vs} \text{ U/L}$$

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 $\xrightarrow{ALT}$ glutamate + pyruvate

Pyruvate + $NADH+H^+$ $\xrightarrow{LDH}$ 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 $\xrightarrow{ALP}$ 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 (ΔA/Min).

Calculation:

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

The molar absorbance (ε) 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 ΔA/min, will give the activity of alkaline phosphatase in U/L.

Normal value: 53 – 128 U/L for male and 42 – 98 U/L for female.

2.2.6. Determination of serum γ- glutamyl transferase

Szasz method

Principle: γ - glutmyl para nitro anilide (GPNA) and glycyl glycine are converted by the action of γ - glutamyl transferase to para nitro aniline and L- γ - 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.

GPNA + glycyl glycine

↓ GGT

P-nitroaniline + L - γ - 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 (ΔA/min) was determined.

Calculations:

Serum GGT activity (IU/L) = ΔA/min X 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 m l 1008U

HRP substrate, Solution A., 1 1 m l 1000SA

HRP substrate, Solution B., 1 1 m l 1000SB

Wash buffer (20X), 50 ml W B - 2 0

Stop solution, 1 0 m l 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 fo serm 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

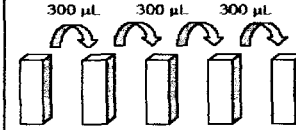
Reagent 2: Antiserum Anti-Ceruloplasmin

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.

Std N°	1	2	3	4	5	6
Dilution	1/7	1/14	1/28	1/56	1/112	0
NaCl (µL) Calibrator (µL)	600 100	300 --	300 --	300 --	300 --	300 --
	300 µL 300 µL 300 µL 300 µL 					
Factor	3.00	1.50	0.75	0.38	0.19	0

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 ratio1: 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.

Contents pipetted into the cuvette	**Blank**	**Calibrator**	**Sample**
NaCl (9g/L)	50 µl	-	-
Calibrator	-	50 µl	-
Dil. Sample	-	-	50 µl
Working reagent	1000 µl	1000 µl	1000 µl

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 $P<0.05$.

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Chapter 3

RESULTS

3.1. Serum bilirubin

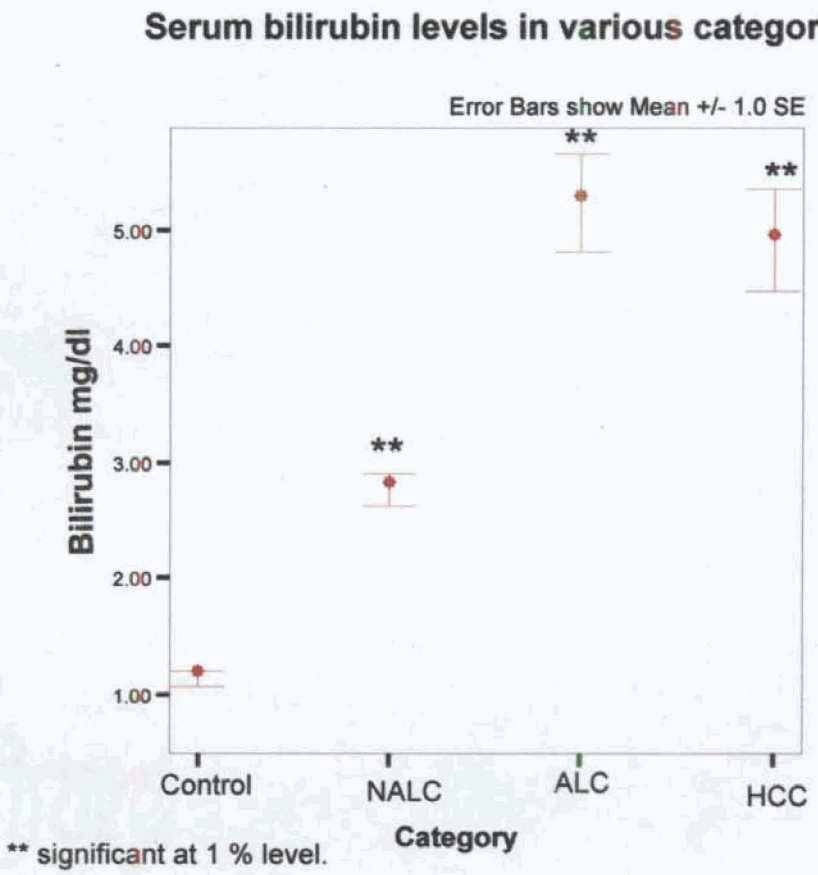


Figure – 3.1.1.

Serum bilirubin mean values in mg/dl in various categories:

Category	Control	NALC	ALC	HCC
Mean	1.1420	2.7685	5.2315	4.9024
SE	.06600	.14052	.41935	.43909
SD	.33001	.80721	2.40899	2.70674
N	25	33	33	38
Min	.69	1.51	1.60	2.00
Max	2.00	4.82	10.23	12.00

Table – 3.1.1.

One way ANOVA of bilirubin values in various categories:

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	**NALC**	-1.6265	.51971	.002	-2.6551	-.5979
	ALC	-4.0895	.51971	.000	-5.1181	-3.0609
	HCC	-3.7604	.50476	.000	-4.7594	-2.7614
NALC	**Control**	1.6265	.51971	.002	.5979	2.6551
	ALC	-2.4630	.48254	.000	-3.4180	-1.5080
	HCC	-2.1339	.46640	.000	-3.0569	-1.2108
ALC	**Control**	4.0895	.51971	.000	3.0609	5.1181
	NALC	2.4630	.48254	.000	1.5080	3.4180
	HCC	.3291	.46640	.482	-.5939	1.2522
HCC	**Control**	3.7604	.50476	.000	2.7614	4.7594
	NALC	2.1339	.46640	.000	1.2108	3.0569
	ALC	-.3291	.46640	.482	-1.2522	.5939

Table – 3.1.2.

Bilirubin groups at different ranges in different categories:

			bilirubin group			Total
			<2	2-4	>4	
Category	Control	Count	24	1		25
		% within Category	96.0%	4.0%		100.0%
	NALC	Count	5	25	3	33
		% within Category	15.2%	75.8%	9.1%	100.0%
	ALC	Count	1	12	20	33
		% within Category	3.0%	36.4%	60.6%	100.0%
	HCC	Count		21	17	38
		% within Category		55.3%	44.7%	100.0%
Total		Count	30	59	40	129
		% within Category	23.3%	45.7%	31.0%	100.0%

Table – 3.1.3.

The mean values of serum bilirubin were increased in NALC, ALC and HCC categories when compared to the control group (Figure – 3.1.1.). The order of increase is NALC < HCC < ALC and the mean values were 1.1420 $\pm$ 0.33001 mg/dl for Control, 2.7685 $\pm$ 0.80721 mg/dl for NALC, 5.23154 $\pm$ 2.40899 mg/dl for ALC and 4.9024 $\pm$ 2.70674 mg/dl for HCC category (Table – 3.1.1.). When compared to the control, the mean differences were - 1.6265, - 4.0895, - 3.7604 for NALC, ALC and HCC respectively and the values were significantly different at 1 % level ($P < 0.01$) (Table – 3.1.2). When NALC was compared with ALC and HCC, the mean values were found significantly increased at 1 % level ($P < 0.01$). The mean values in ALC and HCC categories showed no significant difference (Table – 3.1.2.).

The bilirubin values of 96 % subjects in the control category, 15.2 % of NALC category and 3 % of ALC category were below 2 mg/dl. The bilirubin values of 4 % subjects in the control category, 75.8 % of NALC category, 36.4 % of ALC category and 55.3 % of HCC category were between 2 – 4 mg/dl. The bilirubin values of 9.1 % of NALC category, 60.6 % of ALC category and 44.7 % of HCC category were above 4 mg/dl (Table – 3.1.3.).

3.2. Serum albumin

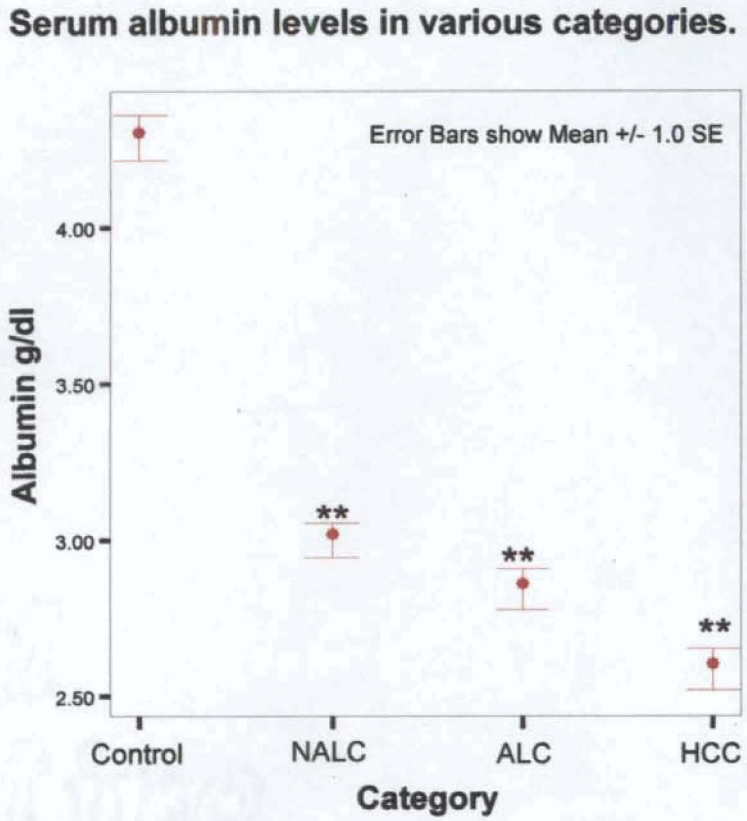


Figure – 3.2.1.

Serum albumin mean values in g/dl in various categories:

Category	Control	NALC	ALC	HCC
Mean	4.2908	2.9970	2.8406	2.5834
SE	.07462	.05667	.06673	.06513
SD	.37311	.32553	.38332	.40150
N	25	33	33	38
Min	3.72	2.15	2.15	2.00
Max	5.10	3.55	4.00	3.20

Table – 3.2.1.

One way ANOVA of albumin values in various categories:

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	NALC	1.2938	.09892	.000	1.0981	1.4896
	ALC	1.4502	.09892	.000	1.2544	1.6460
	HCC	1.7074	.09607	.000	1.5172	1.8975
NALC	Control	-1.2938	.09892	.000	-1.4896	-1.0981
	ALC	.1564	.09185	.091	-.0254	.3381
	HCC	.4135	.08877	.000	.2379	.5892
ALC	Control	-1.4502	.09892	.000	-1.6460	-1.2544
	NALC	-.1564	.09185	.091	-.3381	.0254
	HCC	.2572	.08877	.004	.0815	.4329
HCC	Control	-1.7074	.09607	.000	-1.8975	-1.5172
	NALC	-.4135	.08877	.000	-.5892	-.2379
	ALC	-.2572	.08877	.004	-.4329	-.0815

Table – 3.2.2.

Albumin groups at different ranges in different categories:

			albumin group			Total
			<2.5	2.5-3.5	>3.5	
Category	Control	Count			25	25
		% within Category			100.0%	100.0%
	NALC	Count	1	28	4	33
		% within Category	3.0%	84.8%	12.1%	100.0%
	ALC	Count	7	24	2	33
		% within Category	21.2%	72.7%	6.1%	100.0%
	HCC	Count	19	19		38
		% within Category	50.0%	50.0%		100.0%
Total		Count	27	71	31	129
		% within Category	20.9%	55.0%	24.0%	100.0%

Table – 3.2.3.

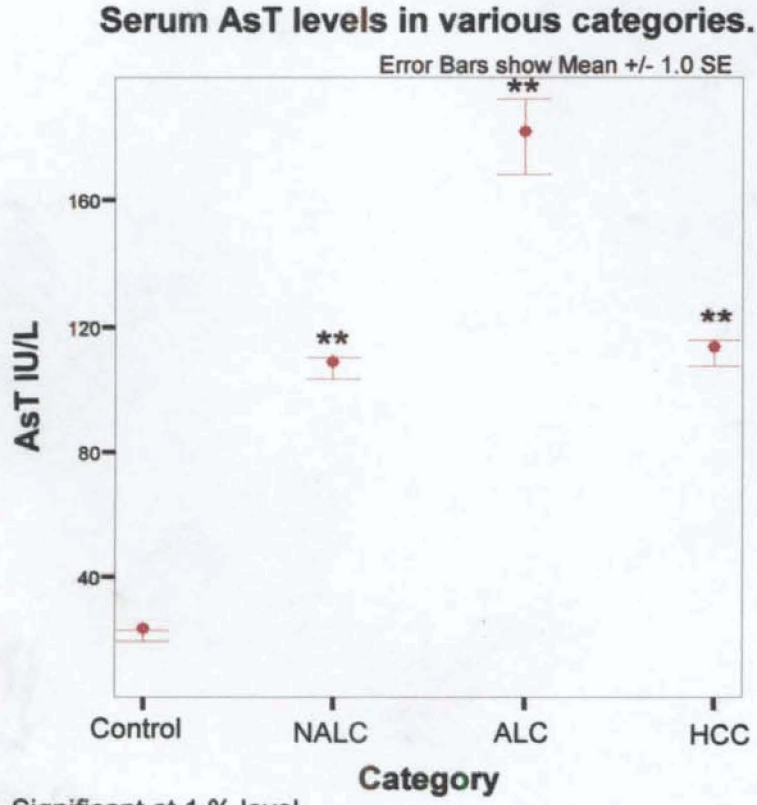
When compared to the control category, the mean values of albumin were found to be decreased in the order of NALC (2.9970 $\pm$ 0.32553 g/dl) > ALC (2.8406 $\pm$ 0.38332 g/dl) > HCC (2.5834 $\pm$ 0.40150 g/dl). The mean value of albumin in the control category is 4.2908 $\pm$ 0.37311 g/dl (Figure – 3.2.1. and Table – 3.2.1.). Mean difference of control and NALC is 1.2938, control and ALC is 1.4502, and control and HCC is 1.7074. All these values showed a significant difference at 1 % level ($P < 0.01$) (Table – 3.2.2.).

When compared to NALC, the mean albumin values of ALC category showed no significant difference, while the HCC category showed a significant decrease at 1 % level ($P < 0.01$). The mean albumin value of HCC category was found to be significantly decreased at 1 % level ($P < 0.01$) when compared to ALC category (Table- 3.2.2.).

The albumin values of 100 % subjects of control category, 12.1 % of NALC category, and 6.1 % of ALC category were above 3.5 g/dl. The albumin values of 84.8 % of NALC category, 72.7 % of ALC category and 50 % HCC category were between 2.5 - 3.5 g/dl. The albumin values of 3 % of NALC category, 21.2 % of ALC category and 50 % of HCC category were below 2.5 g/dl (Table – 3.2.3.).

SERUM ENZYMES

3.3. Serum aspartate transaminase



** Significant at 1 % level.

Figure – 3.3.1.

Serum AsT mean values in IU/L in different categories:

Category	Control	NALC	ALC	HCC
Mean	21.20	106.85	180.45	111.32
SE	1.456	3.360	11.886	4.215
SD	7.280	19.304	68.278	25.983
N	25	33	33	38
Min	8	52	84	66
Max	32	140	341	192

Table – 3.3.1.

One way ANOVA of AsT values in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
AST	Control	NALC	-85.65	10.265	.000	-105.96	-65.33
		ALC	-159.25	10.265	.000	-179.57	-138.94
		HCC	-90.12	9.970	.000	-109.85	-70.38
	NALC	Control	85.65	10.265	.000	65.33	105.96
		ALC	-73.61	9.531	.000	-92.47	-54.74
		HCC	-4.47	9.212	.629	-22.70	13.76
	ALC	Control	159.25	10.265	.000	138.94	179.57
		NALC	73.61	9.531	.000	54.74	92.47
		HCC	69.14	9.212	.000	50.91	87.37
	HCC	Control	90.12	9.970	.000	70.38	109.85
		NALC	4.47	9.212	.629	-13.76	22.70
		ALC	-69.14	9.212	.000	-87.37	-50.91

Table – 3.3.2.

AsT groups at different ranges in different categories·

			AST group			Total
			<100	100-200	>200	
Category	Control	Count	25			25
		% within Category	100.0%			100.0%
	NALC	Count	9	24		33
		% within Category	27.3%	72.7%		100.0%
	ALC	Count	2	24	7	33
		% within Category	6.1%	72.7%	21.2%	100.0%
	HCC	Count	14	24		38
		% within Category	36.8%	63.2%		100.0%
Total		Count	50	72	7	129
		% within Category	38.8%	55.8%	5.4%	100.0%

Table - 3.3.3.

The mean values of AsT were found to be increased in NALC category (106.85 ± 19.304 IU/L), ALC category (180.45 ± 68.278 IU/L) and HCC category (111.32 ± 25.983 IU/L) when compared to the control category (21.20 ± 7.280 IU/L). The order of increase was NALC < HCC < ALC (Figure – 3.3.1. and Table – 3.3.1.). When compared to the control group, the mean differences were - 85.65 for NALC, - 159.25 for ALC and - 90.12 for HCC and the values are highly significant at 1 % level ($P < 0.01$) (Table – 3.3.2.).

The NALC and HCC categories showed no significant change in the mean value of AsT. ALC category showed a significant increase at 1 % level ($P < 0.01$) in the mean value of AsT when compared to NALC and HCC categories (Table – 3.3.2.).

The AsT value of 100 % subjects in control category, 27.3 % of NALC, 6.1 % of ALC category and 36.8 % of HCC category were below 100 IU/L. The AsT value of 72.7 % NALC category, 72.7 % of ALC category and 24 % of HCC category were between 100-200 IU/L. The AsT value of 21.2 % of ALC category, were above 200 IU/L (Table – 3.3.3.).

3.4. Serum alanine transaminase

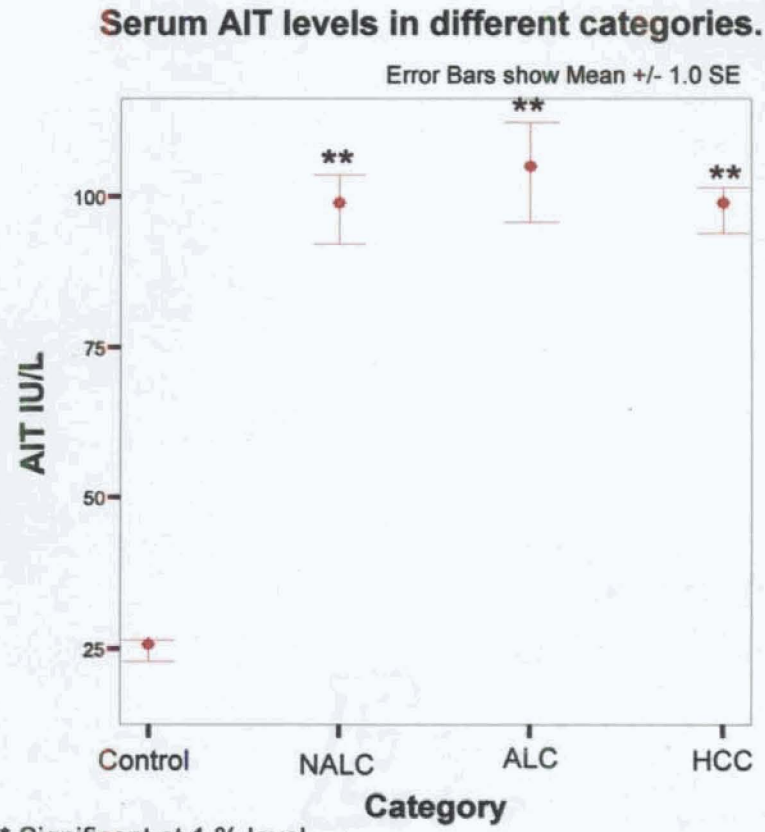


Figure – 3.4.1.

Serum AlT mean values in IU/L in different categories:

Category	Control	NALC	ALC	HCC
Mean	24.56	97.76	104.00	97.58
SE	1.656	5.612	8.251	3.675
SD	8.282	32.238	47.399	22.657
N	25	33	33	38
Min	10	42	51	53
Max	41	170	280	174

Table – 3.4.1

One Way ANOVA of AlT values in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
AlT	Control	NALC	-73.20	8.411	.000	-89.84	-56.55
		ALC	-79.44	8.411	.000	-96.09	-62.79
		HCC	-73.02	8.169	.000	-89.19	-56.85
	NALC	Control	73.20	8.411	.000	56.55	89.84
		ALC	-6.24	7.810	.426	-21.70	9.21
		HCC	.18	7.548	.981	-14.76	15.12
	ALC	Control	79.44	8.411	.000	62.79	96.09
		NALC	6.24	7.810	.426	-9.21	21.70
		HCC	6.42	7.548	.397	-8.52	21.36
	HCC	Control	73.02	8.169	.000	56.85	89.19
		NALC	-.18	7.548	.981	-15.12	14.76
		ALC	-6.42	7.548	.397	-21.36	8.52

Table – 3.4.2.

AlT groups at different ranges in different categories:

			ALT gp			Total
			<100	100-200	>200	
Category	Control	Count	25			25
		% within Category	100.0%			100.0%
	NALC	Count	20	13		33
		% within Category	60.6%	39.4%		100.0%
	ALC	Count	19	13	1	33
		% within Category	57.6%	39.4%	3.0%	100.0%
	HCC	Count	21	17		38
		% within Category	55.3%	44.7%		100.0%
Total		Count	85	43	1	129
		% within Category	65.9%	33.3%	.8%	100.0%

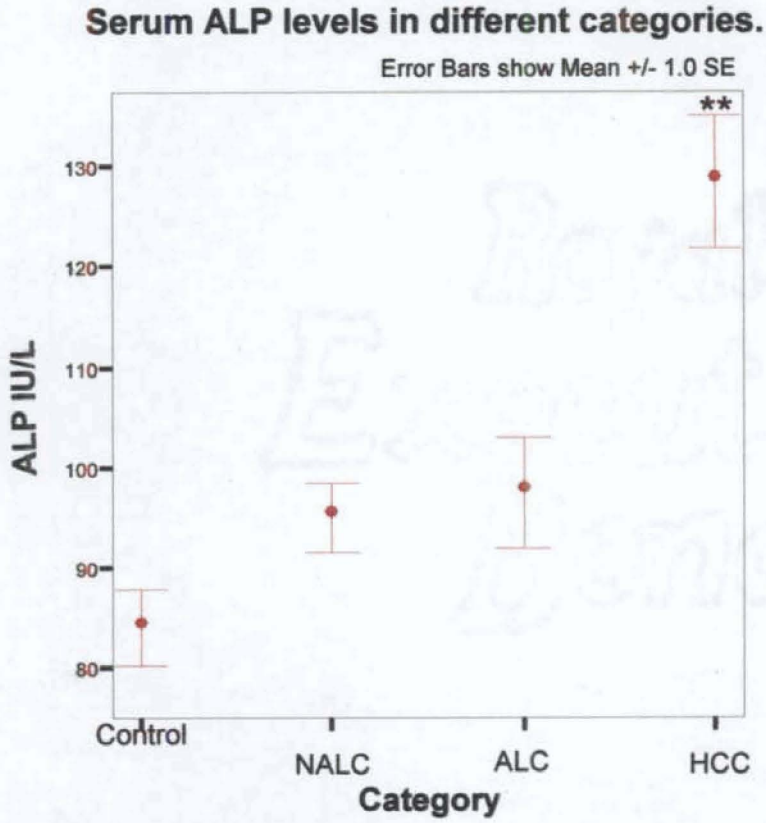
Table – 3.4.3.

When compared to the control group, the mean value of AlT was found to be increased in NALC category (97.76 ± 32.238 IU/L), ALC category (104 ± 47.399 IU/L) and HCC category (97.58 ± 22.657 IU/L). The AlT value of control group was 24.56 ± 8.282 IU/L). The order of increase was HCC < NALC < ALC (Figure – 3.4.1. and Table – 3.4.1.). When compared to the control group, the mean difference was - 73.24 for NALC, - 79.44 for ALC and - 73.024 for HCC and the values showed a significant difference at 1 % level ($P < 0.01$) (Table – 3.4.2.).

When compared with NALC category, the AlT mean values showed no significant difference with ALC and HCC. Similarly the AlT values of ALC and HCC showed no significant difference (Table – 3.4.2.).

AlT values of 100 % of control category, 60.6 % of NALC category, 57.6 % of ALC category and 55.3 % of HCC category were below 100IU/L. The AlT values of 39.4% of NALC category, 39.4 % of ALC category, 44.7 % of HCC category was between 100 – 200 IU/L. The AlT values of 3 % of ALC category was above 200 IU/L (Table – 3.4.3.).

3.5. Serum alkaline phosphatase



** Significant at 1 % level.

Figure – 3.5.1.

Serum ALP mean values in IU/L in different categories:

Category	Control	NALC	ALC	HCC
Mean	84.04	95.00	97.45	128.37
SE	3.733	3.455	5.467	6.644
SD	18.663	19.848	31.408	40.956
N	25	33	33	38
Min	57	40	60	56
Max	128	131	190	206

Table – 3.5.1.

One way ANOVA of ALP values in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
ALP	Control	NALC	-10.96	8.028	.175	-26.85	4.93
		ALC	-13.41	8.028	.097	-29.30	2.47
		HCC	-44.33	7.797	.000	-59.76	-28.90
	NALC	Control	10.96	8.028	.175	-4.93	26.85
		ALC	-2.45	7.454	.742	-17.21	12.30
		HCC	-33.37	7.205	.000	-47.63	-19.11
	ALC	Control	13.41	8.028	.097	-2.47	29.30
		NALC	2.45	7.454	.742	-12.30	17.21
		HCC	-30.91	7.205	.000	-45.17	-16.66
	HCC	Control	44.33	7.797	.000	28.90	59.76
		NALC	33.37	7.205	.000	19.11	47.63
		ALC	30.91	7.205	.000	16.66	45.17

Table – 3.5.2.

ALP groups at different ranges in different categories:

			ALP gp			Total
			<100	100-200	>200	
Category	Control	Count	20	5		25
		% within Category	80.0%	20.0%		100.0%
	NALC	Count	16	17		33
		% within Category	48.5%	51.5%		100.0%
	ALC	Count	21	12		33
		% within Category	63.6%	36.4%		100.0%
	HCC	Count	13	20	5	38
		% within Category	34.2%	52.6%	13.2%	100.0%
Total		Count	70	54	5	129
		% within Category	54.3%	41.9%	3.9%	100.0%

Table – 3.5.3.

The ALP mean value is found to be increased in NALC, ALC and HCC categories when compared to the control category. The order of increase was NALC (95 $\pm$ 19.848 IU/L) < ALC (97.45 $\pm$ 31.408 IU/L) < HCC (128.37 $\pm$ 40.956 IU/L). The ALP mean value of control was 84.04 $\pm$ 18.65 IU/L (Figure – 3.5.1. and Table – 3.5.1.). When compared to control the mean difference of ALP value was - 10.96 for NALC - 13.41 for ALC and - 44.33 for HCC in which only HCC showed a significant change at 1 % level ($P < 0.01$) (Table – 3.5.2.).

No significant change was found between NALC and ALC categories, but a significant change at 1 % level ($P < 0.01$) was observed between NALC and HCC categories and also between ALC and HCC categories (Table – 3.5.2.).

The ALP values of 80 % control category, 48.5 % of NALC category, 63.6 % of ALC category 34.2 % of HCC category were below 100 IU/L. The ALP values of 20 % control category, 51.5 % of NALC category, 36.4 % of ALC category and 52.6 % of HCC category were between 100 – 200 IU/L. The ALP value of 13.2 % of HCC was above 200 IU/L (Table – 3.5.3.).

3.6. Serum γ-Glutamyl transferase

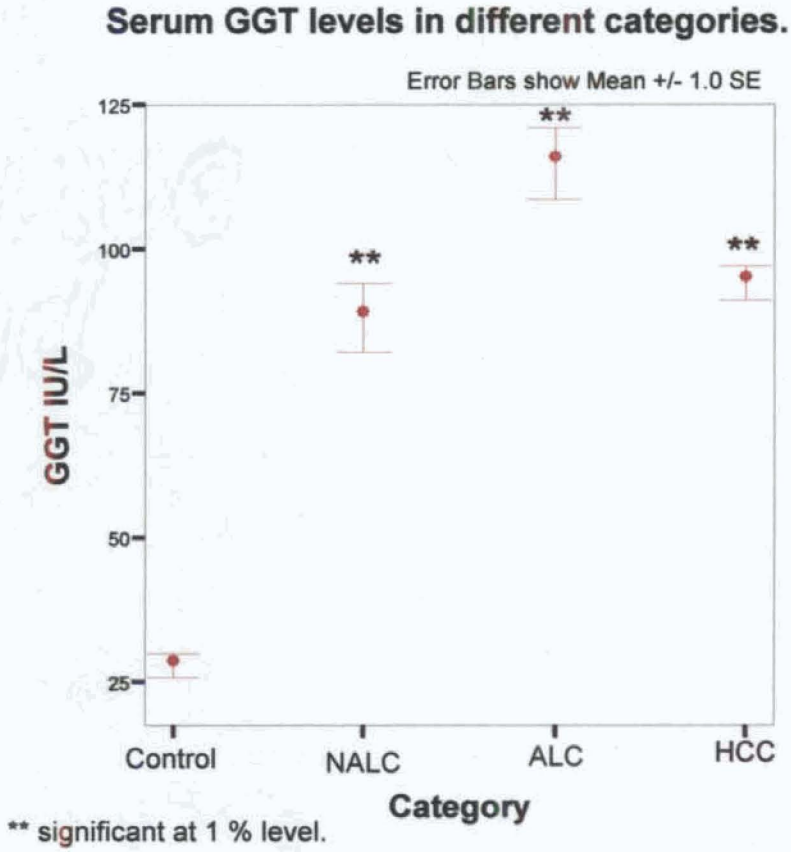


Figure – 3.6.1.

Serum GGT mean values in various categories:

Category	Control	NALC	ALC	HCC
Mean	27.84	88.27	115.03	94.18
SE	2.163	5.989	6.122	2.997
SD	10.815	34.406	35.167	18.474
N	25	33	33	38
Min	12	39	53	56
Max	53	175	200	130

Table – 3.6.1.

One way ANOVA of GGT values in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
GGT	Control	NALC	-60.43	7.228	.000	-74.74	-46.13
		ALC	-87.19	7.228	.000	-101.50	-72.89
		HCC	-66.34	7.020	.000	-80.24	-52.45
	NALC	Control	60.43	7.228	.000	46.13	74.74
		ALC	-26.76	6.711	.000	-40.04	-13.48
		HCC	-5.91	6.487	.364	-18.75	6.93
	ALC	Control	87.19	7.228	.000	72.89	101.50
		NALC	26.76	6.711	.000	13.48	40.04
		HCC	20.85	6.487	.002	8.01	33.68
	HCC	Control	66.34	7.020	.000	52.45	80.24
		NALC	5.91	6.487	.364	-6.93	18.75
		ALC	-20.85	6.487	.002	-33.68	-8.01

Table – 3.6.2.

GGT groups at different ranges in different categories:

			GTT gp			Total
			<50	50-100	>100	
Category	Control	Count	24	1		25
		% within Category	96.0%	4.0%		100.0%
	NALC	Count	2	22	9	33
		% within Category	6.1%	66.7%	27.3%	100.0%
	ALC	Count		14	19	33
		% within Category		42.4%	57.6%	100.0%
	HCC	Count		23	15	38
		% within Category		60.5%	39.5%	100.0%
Total		Count	26	60	43	129
		% within Category	20.2%	46.5%	33.3%	100.0%

Table – 3.6.3.

The mean value of GGT was increased in NALC, ALC and HCC categories when compared to control category. The order of increase was NALC (88.27 $\pm$ 34.406 IU/L) < HCC (94.18 $\pm$ 18.474 IU/L) < ALC (115.03 $\pm$ 35.167 IU/L). The ALP value of control was 27.84 $\pm$ 10.815 IU/L (Figure – 3.6.1. and Table – 3.6.1.). When compared to the control category, NALC, ALC and HCC categories showed a mean difference of - 60.43, - 87.19 and - 66.34 respectively and the values are significantly different at 1 % level ($P < 0.01$) (Table – 3.6.2.).

When compared to NALC and HCC categories, the GGT value of ALC category showed a significant increase at 1 % level ($P < 0.01$), while NALC and HCC categories showed no significant change (Table – 3.6.2.).

The serum GGT value of 96 % of control category and 6.1 % of NALC category were below 50 IU/l. The serum GGT values of 4 % control category, 66.75 % of NALC category, 42.4 % of ALC category and 60.5 % of HCC category were between 50 - 100IU/L. The serum GGT values of 27.3 % of NALC category, 57.6 % of ALC category and 39.5 % 0f HCC category were above 100 IU/L (Table – 3.6.3.).

3.7. Serum AsT/AlT ratio

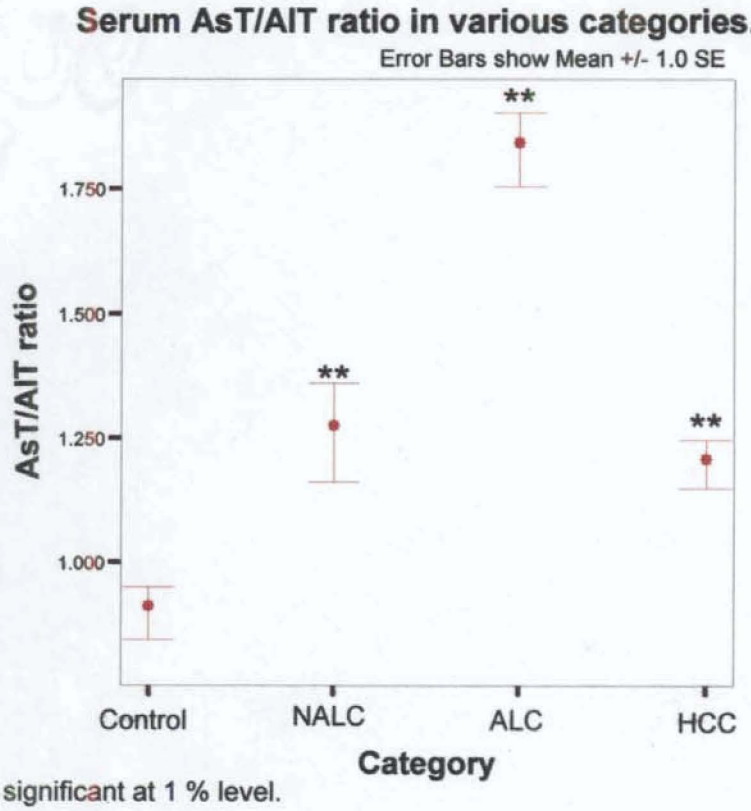


Figure – 3.7.1.

Serum AsT/ AlT ratio mean values in mg/L in different categories:

Category	Control	NALC	ALC	HCC
Mean	.89652	1.26000	1.82848	1.19632
SE	.052946	.098551	.073938	.050648
SD	.264731	.566133	.424743	.312215
N	25	33	33	38
Min	.420	.530	.750	.770
Max	1.410	3.100	2.620	2.240

Table – 3.7.1.

One way ANOVA of AsT/AlT ratio in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
AST/ALT	Control	NALC	-.36348	.109497	.001	-.58019	-.14677
		ALC	-.93196	.109497	.000	-1.14867	-.71526
		HCC	-.29980	.106347	.006	-.51027	-.08932
	NALC	Control	.36348	.109497	.001	.14677	.58019
		ALC	-.56848	.101666	.000	-.76969	-.36728
		HCC	.06368	.098265	.518	-.13079	.25816
	ALC	Control	.93196	.109497	.000	.71526	1.14867
		NALC	.56848	.101666	.000	.36728	.76969
		HCC	.63217	.098265	.000	.43769	.82665
	HCC	Control	.29980	.106347	.006	.08932	.51027
		NALC	-.06368	.098265	.518	-.25816	.13079
		ALC	-.63217	.098265	.000	-.82665	-.43769

Table – 3.7.2.

AsT/AlT ratio groups at different ranges in different categories:

			AST/ALT gp			Total
			<1	1-2	>2	
Category	Control	Count	16	9		25
		% within Category	64.0%	36.0%		100.0%
	NALC	Count	10	21	2	33
		% within Category	30.3%	63.6%	6.1%	100.0%
	ALC	Count	2	20	11	33
		% within Category	6.1%	60.6%	33.3%	100.0%
	HCC	Count	12	25	1	38
		% within Category	31.6%	65.8%	2.6%	100.0%
Total		Count	40	75	14	129
		% within Category	31.0%	58.1%	10.9%	100.0%

Table – 3.7.3.

The AsT/AlT ratio of all the three disease categories were found to be increased when compared to the control group. The order of increase was HCC (1.19632 $\pm$ 0.312215) < NALC (1.260 $\pm$ 0.56613) < ALC (1.82848 $\pm$ 0.424743). The AsT/AlT ratio of control group was 0.89652 $\pm$ 0.26731 (Figure – 3.7.1. and Table – 3.7.1.). When compared to the control category, NALC, ALC and HCC showed a significant increase at 1 % level ($P < 0.01$) and the mean differences were - 0.36348, - 0.93196 and - 0.29980 respectively (Table – 3.7.2.).

When compared to NALC and HCC categories, ALC category showed a significant increase in AsT/AlT ratio at 1 % level ($P < 0.01$), while NALC and HCC categories showed no significant change (Table – 3.7.2.).

The AsT/Alt ratio of 64 % control category, 30.3 % of NALC category, 6.1 % of ALC category and 31.6 % of HCC category were below 1. The AsT/AlT ratio of 36 % of control category, 63.6 % of NALC category, 60.6 % of ALC category and 65.8 % of HCC category were between 1 - 2. The AsT/AlT ratio of 6.1 % NALC, 33.3 % ALC, 2.6 % HCC categories were above 2 (Table – 3.7.3.).

SERUM ACUTE PHASE PROTEINS

3.8. Serum C-reactive protein

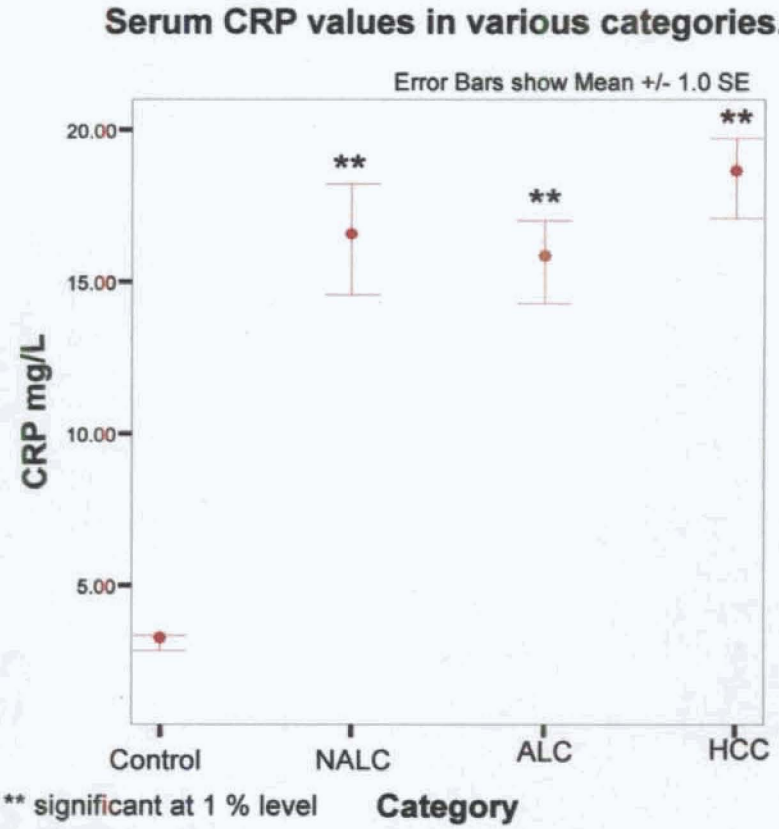


Figure – 3.8.1.

Serum CRP mean values in mg/L in different categories:

Category	Control	NALC	ALC	HCC
Mean	3.0436	16.3733	18.9736	18.3895
SE	.25135	1.84887	3.51504	1.31355
SD	1.25677	10.6209	20.1924	8.09724
N	25	33	33	38
Min	1.15	3.50	3.80	5.20
Max	7.32	40.70	122.75	32.00

Table – 3.8.1.

One way ANOVA of CRP values in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
CRP	Control	NALC	-13.3297	2.12469	.000	-17.5348	-9.1247
		ALC	-12.5823	2.12469	.000	-16.7873	-8.3773
		HCC	-15.3459	2.06356	.000	-19.4299	-11.2618
	NALC	Control	13.3297	2.12469	.000	9.1247	17.5348
		ALC	.7474	1.97272	.705	-3.1568	4.6517
		HCC	-2.0161	1.90673	.292	-5.7898	1.7575
	ALC	Control	12.5823	2.12469	.000	8.3773	16.7873
		NALC	-.7474	1.97272	.705	-4.6517	3.1568
		HCC	-2.7636	1.90673	.150	-6.5372	1.0101
	HCC	Control	15.3459	2.06356	.000	11.2618	19.4299
		NALC	2.0161	1.90673	.292	-1.7575	5.7898
		ALC	2.7636	1.90673	.150	-1.0101	6.5372

Table – 3.8.2.

CRP groups at different ranges in different categories:

			CRP gp			Total
			<5	5-10	>10	
Category	Control	Count	24	1		25
		% within Category	96.0%	4.0%		100.0%
	NALC	Count	7	5	21	33
		% within Category	21.2%	15.2%	63.6%	100.0%
	ALC	Count	5	4	24	33
		% within Category	15.2%	12.1%	72.7%	100.0%
	HCC	Count	1	6	31	38
		% within Category	2.6%	15.8%	81.6%	100.0%
Total		Count	37	16	76	129
		% within Category	28.7%	12.4%	58.9%	100.0%

Table – 3.8.3.

The mean CRP value was found to be increased in NALC (16.3733 ± 10.6209 mg/L), ALC (18.9736 ± 20.1924 mg/L) and HCC (18.38995 ± 8.09724 mg/L) categories when compared to the control category (3.0436 ± 1.25677 mg/L) and the order of increase was NALC > ALC > HCC (Figure – 3.8.1. and Table – 3.8.1). When compared to the control, the mean difference values were - 13.3297 for NALC, -12.5823 for ALC and -15.3459 for HCC and the values were significant at 1 % level ($P < 0.01$) (Table – 3.8.2.).

The mean values of NALC, ALC and HCC showed no statistical significance (Table – 3.8.2.).

The CRP values of 96 % of control category, 21.2 % of NALC, 15.2 % of ALC category and 2.6 % of HCC category were below 5 mg/L, while 4 % of control category, 1.2 of NALC category, 12.1 % of ALC category and 15.8 % of HCC category were within the range of 5 – 10 mg/L. The CRP values of 63.6 % of NALC category, 72.7 % of ALC category and 81.6 % of HCC category were above 10 mg/L (Table – 3.8.3.).

3.9. Serum transferrin

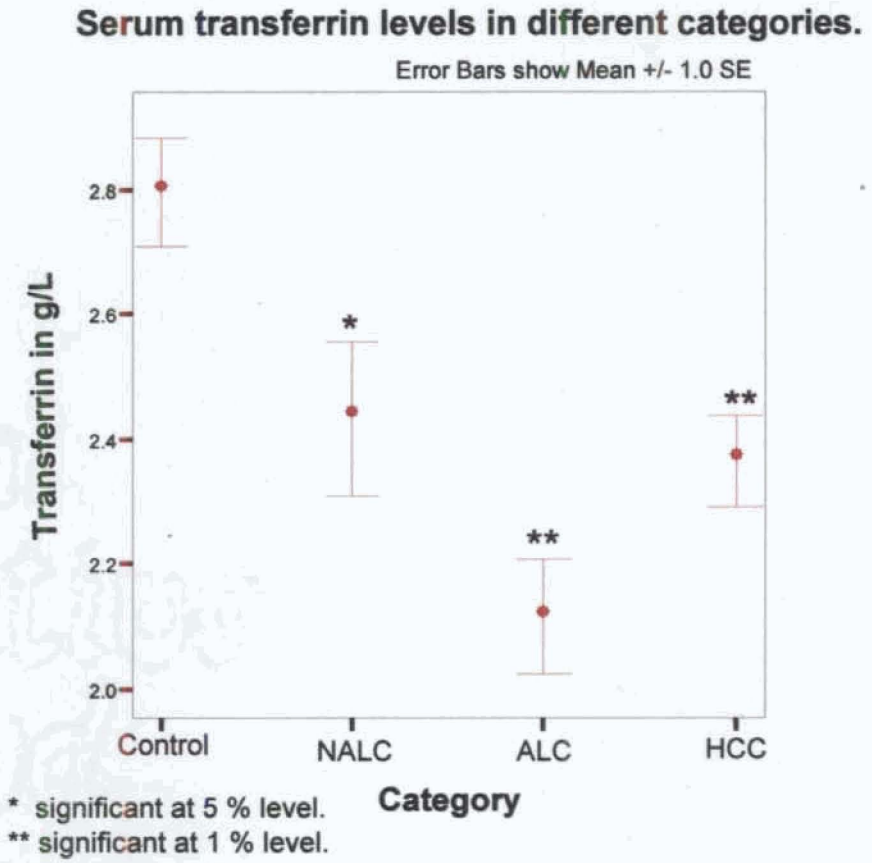


Figure – 3.9.1.

Serum transferrin levels in g/L in different categories:

Category	Control	NALC	ALC	HCC
Mean	2.796	2.433	2.115	2.365
SE	.0861	.1231	.0930	.0746
SD	.4306	.7074	.5340	.4601
N	25	33	33	38
Min	2.1	1.0	1.1	1.4
Max	3.5	4.0	3.1	3.2

Table – 3.9.1.

One way ANOVA of transferrin values in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Transferrin	Control	NALC	.363	.1451	.014	.076	.650
		ALC	.681	.1451	.000	.394	.968
		HCC	.431	.1409	.003	.152	.710
	NALC	Control	-.363	.1451	.014	-.650	-.076
		ALC	.318	.1347	.020	.052	.585
		HCC	.069	.1302	.599	-.189	.326
	ALC	Control	-.681	.1451	.000	-.968	-.394
		NALC	-.318	.1347	.020	-.585	-.052
		HCC	-.250	.1302	.058	-.507	.008
	HCC	Control	-.431	.1409	.003	-.710	-.152
		NALC	-.069	.1302	.599	-.326	.189
		ALC	.250	.1302	.058	-.008	.507

Table – 3.9.2.

Transferrin groups at different ranges in different categories:

			Trans gp			Total
			<2	2-3	>3	
Category	Control	Count		17	8	25
		% within Category		68.0%	32.0%	100.0%
	NALC	Count	10	15	8	33
		% within Category	30.3%	45.5%	24.2%	100.0%
	ALC	Count	12	19	2	33
		% within Category	36.4%	57.6%	6.1%	100.0%
	HCC	Count	8	25	5	38
		% within Category	21.1%	65.8%	13.2%	100.0%
Total		Count	30	76	23	129
		% within Category	23.3%	58.9%	17.8%	100.0%

Table – 3.9.3.

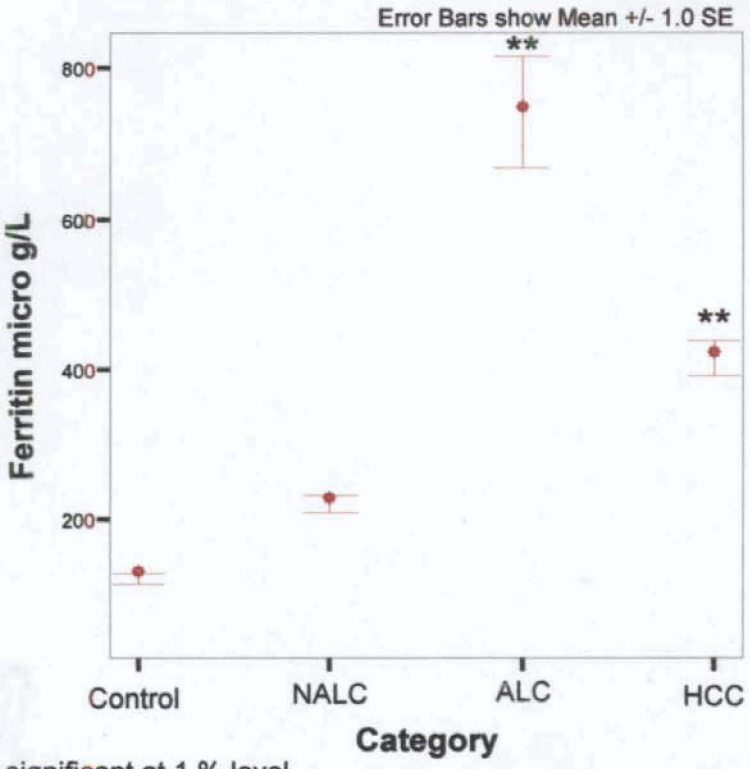
The mean transferrin values of NALC, ALC and HCC categories were found to be decreased when compared to the control group. The order of decrease was NALC (2.433 $\pm$ 0.7074 g/l) > HCC (2.365 $\pm$ 0.4601 g/l) > ALC (2.115 $\pm$ 0.5340 g/l). The mean value of control was 2.796 $\pm$ 0.4306 (Figure – 3.9.1. and Table – 3.9.1.). The mean differences were 0.363, 0.681 and 0.431 respectively for NALC, ALC and HCC categories when compared to the control category. The mean values of ALC and HCC categories were significantly different at 1 % level ($P < 0.01$) and the mean value of NALC showed a significant difference at 5 % level ($P < 0.05$) when compared to the control category (Table – 3.9.2.).

The mean transferrin values of ALC category showed a significant decrease at 5 % level ($P < 0.05$) when compared with that of NALC category, while no significant difference was observed when compared to HCC. Similarly HCC and NALC categories showed no significant change in the mean transferrin values (Table – 3.9.2.).

The serum transferrin value of 32 % of control category, 24.2 % of NALC category, 6.1 % of ALC category and 13.2 % of HCC category were above 3 g/dl. The serum transferrin value of 68.5 % of control category, 45.5 % of NALC category, 57.6 % of ALC category and 65.8 % of HCC category were 2 – 3 g/l. The serum transferrin value of 30.3 % of NALC, 36.4 % of ALC and 21.15 of HCC categories were below 2 g/l (Table – 3.9.3.).

3.10. Serum ferritin

Serum ferritin levels in different categories.



** significant at 1 % level.

Figure – 3.10.1.

Serum ferritin mean values in µg/L in different categories:

Category	Control	NALC	ALC	HCC
Mean	121.72	221.06	741.70	414.71
SE	6.769	10.617	74.382	22.842
SD	33.843	60.988	427.293	140.809
N	25	33	33	38
Min	60	39	250	209
Max	190	322	1850	800

Table – 3.10.1.

One way ANOVA of ferritin values in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Ferritin	Control	NALC	-99.34	61.490	.109	-221.04	22.36
		ALC	-619.98	61.490	.000	-741.67	-498.28
		HCC	-292.99	59.721	.000	-411.18	-174.80
	NALC	Control	99.34	61.490	.109	-22.36	221.04
		ALC	-520.64	57.092	.000	-633.63	-407.64
		HCC	-193.65	55.182	.001	-302.86	-84.44
	ALC	Control	619.98	61.490	.000	498.28	741.67
		NALC	520.64	57.092	.000	407.64	633.63
		HCC	326.99	55.182	.000	217.77	436.20
	HCC	Control	292.99	59.721	.000	174.80	411.18
		NALC	193.65	55.182	.001	84.44	302.86
		ALC	-326.99	55.182	.000	-436.20	-217.77

Table – 3.10.2.

Ferritin groups at different ranges in different categories:

			Ferritin gp			Total
			<250	250-350	>350	
Category	Control	Count	25			25
		% within Category	100.0%			100.0%
	NALC	Count	25	8		33
		% within Category	75.8%	24.2%		100.0%
	ALC	Count		2	31	33
		% within Category		6.1%	93.9%	100.0%
	HCC	Count	3	11	24	38
		% within Category	7.9%	28.9%	63.2%	100.0%
Total		Count	53	21	55	129
		% within Category	41.1%	16.3%	42.6%	100.0%

Table – 3.10.3.

The mean ferritin values of NALC (221.06 ± 60.988 µg/l), ALC (741.70 ± 427.293 µg/l) and HCC (414.71 ± 140.809 µg/l) categories were found to be increased when compared to the control category (121. 72 ± 33.843 µg/l). The order of increase was NALC < HCC < ALC (Figure – 3.10.1. and Table – 3.10.1.). The mean difference values of NALC, ALC and HCC categories were - 99.34, - 619.98 and - 292.99 respectively when compared to the control group. When compared to control category, a significant increase at 1 % level ($P < 0.01$) was found in serum ferritin value of ALC category and HCC category, while the increase in serum ferritin value in NALC showed no statistical significance (Table – 3.10.2.).

When compared to the NALC category the serum ferritin values were found to be significantly increased at 1 % level ($P < 0.01$) in ALC and HCC categories. When compared to HCC category, ALC showed a significant increase at 1 % level ($P < 0.01$) in serum ferritin level (Table – 3.10.2.).

The serum ferritin values of 100 % of control category, 75.8 % of NALC category, and 7.9 % of HCC category were below 250 µgm/l. The serum ferritin level of 24.2 % of NALC category, 6.1 % of ALC category and 28.9 % of HCC category were between 250 - 350 µg/l. The serum value of 93.9 % of ALC category and 63.2 % of HCC category were above 350 µg/l (Table - 3.10.3.).

3.11. Serum ceruloplasmin

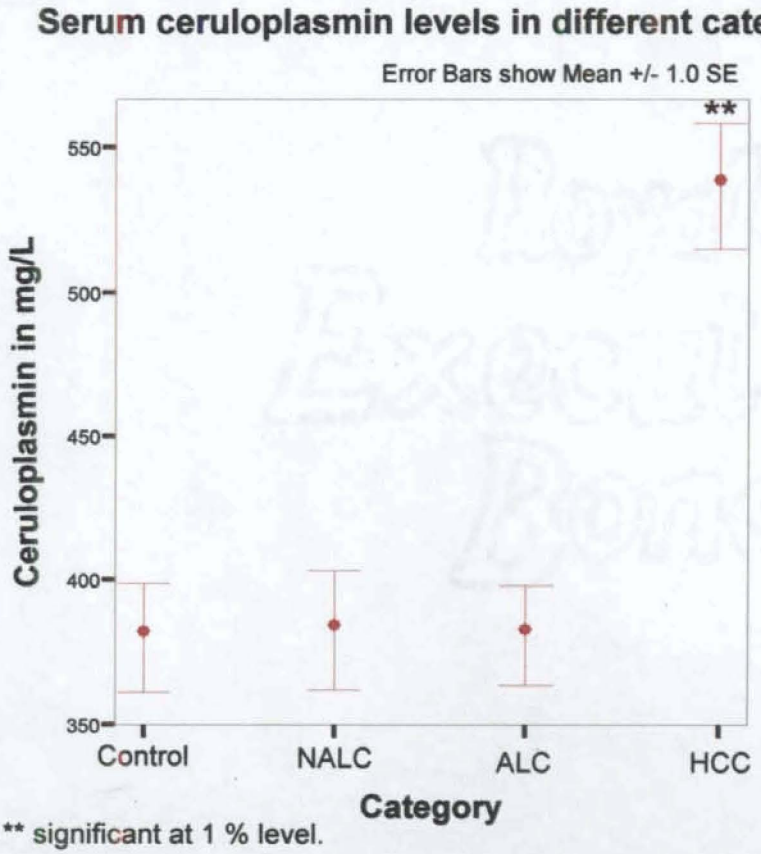


Figure – 3.11.1.

Serum ceruloplasmin mean values in mg/L in different categories:

Category	Control	NALC	ALC	HCC
Mean	379.80	382.30	380.85	536.42
SE	19.052	20.726	17.474	22.019
SD	95.261	119.061	100.379	135.734
N	25	33	33	38
Min	220	194	230	300
Max	580	630	590	802

Table – 3.11.1.

One way ANOVA of ceruloplasmin values in different categories:

Dependent Variable	(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Ceruloplasmin	Control	NALC	-2.50	30.698	.935	-63.26	58.25
		ALC	-1.05	30.698	.973	-61.80	59.71
		HCC	-156.62	29.815	.000	-215.63	-97.61
	NALC	Control	2.50	30.698	.935	-58.25	63.26
		ALC	1.45	28.502	.959	-54.95	57.86
		HCC	-154.12	27.549	.000	-208.64	-99.60
	ALC	Control	1.05	30.698	.973	-59.71	61.80
		NALC	-1.45	28.502	.959	-57.86	54.95
		HCC	-155.57	27.549	.000	-210.09	-101.05
	HCC	Control	156.62	29.815	.000	97.61	215.63
		NALC	154.12	27.549	.000	99.60	208.64
		ALC	155.57	27.549	.000	101.05	210.09
		HCC	-659.394	30.4766	.000	-719.711	-599.077

Table – 3.11.2.

Ceruloplasmin groups at different ranges in different categories:

			Cerulo gp			Total
			<250	250-350	>350	
Category	Control	Count	2	8	15	25
		% within Category	8.0%	32.0%	60.0%	100.0%
	NALC	Count	4	12	17	33
		% within Category	12.1%	36.4%	51.5%	100.0%
	ALC	Count	3	10	20	33
		% within Category	9.1%	30.3%	60.6%	100.0%
	HCC	Count		3	35	38
		% within Category		7.9%	92.1%	100.0%
Total		Count	9	33	87	129
		% within Category	7.0%	25.6%	67.4%	100.0%

Table – 3.11.3.

The mean values of ceruloplasmin were found to be increased in NALC, ALC and HCC categories, when compared to the control category. The order of increase was ALC (380.85 $\pm$ 100.379 mg/l) < NALC (382.30 $\pm$ 119 mg/l) < HCC (536.42 $\pm$ 135.734 mg/l). The ceruloplasmin value of control category was 379.80 $\pm$ 95.261 mg/l (Figure – 3.11.1. and Table – 3.11.1.). The mean differences were - 2.50 for NALC, - 1.05 for ALC and - 156.62 for HCC categories. When compared to the control, HCC category showed a significant increase at 1 % level ($P < 0.01$), while NALC and ALC categories showed no statistical significance (Table – 3.11.2.).

The HCC category showed a significant increase at 1 % level ($P < 0.01$) when compared to NALC category and ALC category. The values of NALC and ALC were not statistically significant (Table – 3.11.2.).

The serum ceruloplasmin level of 8 % subjects of control category, 12.1 % of NALC category and 9.1 % ALC category were below 250 mg/L. The ceruloplasmin value of 32 % subjects of control category, 36.4 % of NALC category 30.3 % of ALC category and 7.9 % of HCC category were between 250 and 350 mg/L. Ceruloplasmin level of 60 % of the control category, 51.5 % of NALC category, 60.6 % of ALC category and 92.1 % of HCC category were above 350 mg/L (Table – 3.11.3.).

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Chapter 4

DISCUSSION

4.1. Bilirubin and albumin

In the present study bilirubin level was found to be increased in NALC, ALC and HCC categories when compared to the control category. It is well known that the level of bilirubin increases in liver diseases. Huseyin *et al.*, (2006) reported in their study, an increased level of total bilirubin in liver cirrhosis and hepatocellular carcinoma. The study of Alan *et al.*, (1994) showed an increased level of total bilirubin in liver cirrhosis. Many other studies also showed increased level of bilirubin in cirrhosis and hepatocellular carcinoma, which is in accordance with our results.

Bilirubin is formed mainly in the spleen and is transported to liver for conjugation process. Conjugation step takes place in hepatic endoplasmic reticulum in the presence of enzyme bilirubin UDP-glucuronyltransferase. Conjugated bilirubin (mono or di glucuronates) is more soluble and easily gets eliminated through bile in normal case. The hepatocytes microtubular system, hepatic bile salt excretion and membrane carrier proteins, appear to facilitate the excretion of bilirubin glucuronates into bile (Berk and Noyer, 1994). They also reported that the process of biliary excretion, the rate - limiting step in bilirubin transport is susceptible to damage from a variety of acquired liver diseases, which leads to an increased concentration of conjugated bilirubin. In cirrhosis, the canaliculi are abnormal and the relationship between the liver cells and the canaliculi is destroyed. As a result, the liver is not able to eliminate toxic substances normally, and they can accumulate in the body (Bruix and Sherman (2005). So, there is regurgitation of conjugated bilirubin from liver to the circulation. A small fraction of conjugated bilirubin undergo deconjugation, and another small fraction covalently bind with albumin to form delta bilirubin, which is very slowly cleared from the plasma, resulting in an increased plasma bilirubin in liver diseases (Bosma *et al.*, 1994, 1995). Fuad (2003) reported conjugated hyperbilirubinemia in liver cirrhosis and in bile duct obstructing tumors. Acetaldehyde formed by the enzymatic oxidation of

alcohol in the liver is said to play a major role in the development of alcoholic liver cell injury (Jenkins, 1984). Among the diseased category, ALC and HCC categories showed a significant increase in bilirubin level when compared to NALC category, probably due to the toxic effect of ethanol in the liver in ALC category, and tumor growth in HCC category, that might have blocked the removal of bilirubin through bile in that category.

Albumin is the most commonly measured serum protein and is synthesized exclusively by the liver. Serum level of albumin is an indication of the synthetic capacity of the liver. The rate of synthesis varies depending upon the hormonal environment, nutritional status, age and other local factors. Prijatmoko *et al.*, (1993) showed a decreased level of albumin with increasing severity of cirrhosis, with an increase in total body water, whereas total body protein decreased with a significant decrease in albumin. Albumin is found to be decreased in response to inflammation and cellular damage of liver (Baumann *et al.*, 1983, 1984; Beaudet *et al.*, 1982). Ramadori *et al.*, (1985) and Dinarello (1988) reported that output of liver derived proteins, albumin and transferrin, diminishes following infection, inflammation and injury.

In liver diseases, hypoalbuminemia is frequently present because of decreased production and sinusoidal leakage of albumin in patients with portal hypertension. The patterns of plasma protein alterations seen in liver disease depend on the type, severity and duration of liver injury. Hypoalbuminaemia is reported in cirrhosis (Luo *et al.*, 2002) and hepatocellular carcinoma (Dufour, 2000). In cirrhosis, hepatic synthesis of albumin is reduced. Loss of albumin into ascitic fluid also seems to be responsible for the decrease in albumin in many cases. In our study also there is decreased level of albumin in NALC, ALC and HCC categories, when compared to the control category. This may be due to the above multifactorial effects like sinusoidal leakage, reduced synthesis and cell necrosis in liver diseases.

Albumin is a ubiquitous protein synthesized only by hepatocytes. It has been reported that the expression of ALB gene is reduced in various liver diseases and the degree of reduction in the hepatic ALB mRNA level is generally correlated with the severity of the disease (Osaki *et al.*, 1991). The study of Shi–Min *et al.*, (2004) showed that hepatic albumin mRNA in tumor tissue was lower than non tumor tissue. Another study shows that in inflammatory conditions, albumin synthesis is decreased by direct inhibition of cytokines, which are released during acute phase response (Ryffel *et al.*, 1994). It is reflected in our study that the level of albumin is low in HCC category when compared to NALC and ALC categories.

Northern hybridization studies of Annoni *et al.*, (1990) showed that, despite the presence of inflammation and fibrosis, the ALB mRNA levels of alcoholics were similar to the controls. Alcohol actually increases ALB mRNA in alcoholics, but the inhibition of albumin synthesis by alcohol is also reported (Dufour *et al.*, 2000). There are many studies showing that there is a reduction in the level of albumin in alcoholic liver disease. Sivagurunathan *et al.*, (2006) observed in their work a significantly low level of albumin in 80 % of cirrhotic patients with chronic alcohol abuse. Bilirubin values of these patients are also reported to be increased significantly. All these studies support our findings that the albumin level of ALC category is very low than NALC category, probably due to the inhibition of albumin synthesis by alcohol.

SERUM ENZYMES

4.2. Aminotransferases (Aspartate transaminase and alanine transaminase)

In the present study, serum AsT and AlT values were significantly increased in NALC, ALC and HCC categories when compared to the control group. Injury to liver, whether acute or chronic, eventually results in an increase in serum

aminotransferases (Cohen and Kaplan, 1979). Both aminotransferases are highly concentrated in liver. AsT is also diffusely present in the heart, skeletal muscles, kidneys, brain and red blood cells and AlT has low concentrations in skeletal muscles and kidneys (Wroblewski, 1958). An increase in serum AlT levels is therefore more specific for liver damage. In our results, increased AlT in all diseased categories indicates that there was severe liver damage.

It is reported that the liver AlT is localized solely in the cellular cytoplasm, whereas AsT is both cytosolic and mitochondrial (Rej, 1989). Zone 3 of the hepatic acinus has a higher concentration of AsT and damage to this zone may result in greater alteration to AsT levels (Edoardo *et al.*, 2005). Aminotransferase clearance is carried out within the liver by sinusoidal cells (Kamimoto *et al.*, 1985). The half life in the circulation is about 47 hours for AlT, about 17 hours for AsT, and an average of 87 hours for mitochondrial AsT (Dufour *et al.*, 2000). About 80 % of total activity of AsT is found to be by mitochondrial fraction. In the present study, AsT increase is higher than that of AlT indicating that the damage of liver tissue leads to the release of both cytoplasmic and mitochondrial enzymes.

Many studies showed increased levels of aminotransferase in different diseases. In ischaemic or toxic liver injury and acute hepatic injury, the level is found to be greater than 75 times the upper reference limit (Dufour *et al.*, 2000). Siagris *et al.*, (2006) in their study showed an increased level of AsT and AlT in chronic hepatitis patients. Sivagurunathan (2006) reported a significant increase in liver marker enzymes in liver cirrhotic patients. Our results also revealed the same, where the increase in AsT was approximately nine times in ALC category, and approximately five times both in NALC and HCC categories when compared to the control category.

Casaril *et al.*, (2000) observed that Fe^{2+} together with alcohol intensified the liver fibrosis due to the additional activating effect of ethanol on the acceleration of redox reactions within hepatocytes. These redox reactions lead to a cytosolic pH decrease with increased release of free Fe^{2+} ions from ferritin. These reactions may induce lipid peroxidations and a consequent damage to the organelle membrane. These cellular alterations may result in siderogenesis. The study of Fabris *et al.*, (1993) showed that patients with acute hepatitis had significantly higher concentrations of lipid peroxide compared with patients with chronic liver disease. In hepatocellular carcinoma, they could not find evidence of lipid peroxide liberation greater than that found in mild form of liver disease. They also found that the highest lipid peroxide concentration in patients with acute hepatitis were due to drugs or alcohol. Tsukamoto (2001) reported that the primary factors involved in the development of alcoholic liver disease are acetaldehyde, oxidative stress, hypoxia, membrane changes and immune response. All these factors indicate that in alcoholic liver disease, the intensity of liver necrosis is very high. Alcohol also appears to induce the expression of mitochondrial AsT on the surface of hepatocytes (Zhou *et al.*, 1998). It is reported that alcoholic hepatitis is associated with increased plasma activities of mitochondrial AsT and it is proposed that mitochondrial AsT is a marker of chronic alcoholism (Nalpas *et al.*, 1986; Okuno *et al.*, 1988). Alcohol also leads to the release of AsT from other tissues, as AsT is present in other tissues also. It is known that increased level of aminotransferases is an indication for liver necrosis. In the present study, among the diseased category, serum values of AsT and AlT are higher in ALC category when compared to NALC and HCC categories, which may be due to these effects.

The level of AlT in the blood is increased in conditions where hepatocytes are damaged or dead. As cells are damaged, AlT leaks out into the blood stream. All types of hepatitis and cirrhosis have been reported to cause liver damage that can lead to elevations in the serum AlT activity (Cohen and Kaplan 1979). AlT is a

cytosolic enzyme of the liver, which can be increased in cases of liver cell death resulting also from other causes such as shock and drug toxicity. The level of AlT may also be correlated roughly with the degree of cell death or inflammation. In our study, it is noted that there is an increase in serum AlT activity in diseased categories approximately 4 times than that of the control category, and it was also noted that the rate of increase of AlT is less than that of AsT. It has been found that in cirrhosis and malnutrition, there is greater decrease in cytoplasmic AlT than cytoplasmic AsT (Ludwig and Kaplowitz, 1980). Malnutrition is common in cirrhosis and in carcinoma. Reuler (1985) reported that thiamine and pyridoxine deficiency is found to be common in chronic alcoholics. The causes of thiamine and pyridoxine deficiency in alcoholism include poor diet and reduced intestinal absorption. Pyridoxal deficiency is also related to ethanol metabolism in the liver because the production of acetaldehyde results in the displacement of pyridoxal phosphate from albumin, followed by urinary excretion of the unbound vitamin. Pyridoxal phosphate is very important for AlT activity. So, the deficiency of pyridoxine may be a reason for reduced activity of AlT than AsT in alcoholic liver cirrhosis.

In our study, the AsT/AlT ratio is found to be increased in all diseased categories than the control category. The study of Cohen and Kaplan (1979) and Correia *et al.*, (1981) described that AsT/AlT ratio of patients with alcoholic liver disease is above 2, and in patients with non alcoholic liver disease, the AsT/AlT ratio was below 1. They also reported that there was a very low AsT/AlT ratio in toxic and viral hepatitis, chronic active hepatitis and cholestasis jaundice.

Numerous studies have suggested that the serum AsT/AlT ratio may help discriminate between alcoholic and non alcoholic liver disease. In the present study, the mean AsT/AlT ratio of ALC category is 1.8 and it is highly significant when compared to that of NALC and HCC categories. Most of the cases in ALC category

showed the value above 2, while most of the NALC and HCC category showed the value below 1. The AsT/AlT ratio below 1 has been reported in patients with non alcoholic steatohepatitis (Diehl *et al.*, 1988; Bacon *et al.*, 1994). Chedid *et al.*, (1991) and Cohen and Kaplan, (1979) reported that AsT/AlT ratio often exceeds 2 in patients with alcoholic liver disease. Our study also relates the work of Cohen and Kaplan (1979) and Correia *et al.*, (1981), in which they showed that most patients with alcoholic liver disease have ratios >2, whereas, most patients with non alcoholic liver cirrhosis had a ratio of <1. Many studies showed that the AsT/AlT ratio is often greater than 1 in cirrhosis (Sharp 1995; Williams and Hoofnagle, 1988; Park *et al.*, 2000; Giannini *et al.*, 2003) which is in accordance with our results.

Clearance of liver enzymes from plasma occurs at variable rates. The half life of AlT is 47 hours and the half life of cytosolic AsT is 17 hours which means more AsT is cleared from liver and the much longer half life of AlT leads to higher activities of AlT than AsT in most forms of hepatocellular injury. In many cases of liver inflammation AsT and AlT activities are elevated roughly in the ratio of 1:1. But in some conditions like alcoholic hepatitis and chronic hepatitis (infections), the serum AsT levels have been reported to be elevated higher than the serum AlT levels (Khokhar, 2003) that lead to the increase in AsT/AlT ratio to above 1.

4.3. Alkaline phosphatase (ALP)

In the present study, the level of ALP was found to be significantly increased in HCC category when compared to control, NALC and ALC categories. NALC and ALC categories showed only a slight elevation in ALP value than the control category. Increased synthesis of ALP in diseased human liver was reported by Moss (1994). Increased level of ALP has been reported in cirrhotic patients (Sivagurunathan *et al.*, 2006).

Increased level of ALP is an indication of hepatobiliary disease. ALP is a membrane bound glycoprotein enzyme on the canalicular membrane of the hepatocytes (Ludwig, 1980). The fragments of hepatocytes membrane rich in ALP activity have been detected in plasma of patients with cholestasis. This is due to the result of the fragmentation by bile acids. Schlaeger *et al.*, (1982) in their *in vitro* studies demonstrated the membrane fragmentation by bile acids. It has been found that fasting plasma bile acid concentration is elevated in hepatocellular carcinoma. Elevated levels of alkaline phosphatase are seen in primary or secondary liver cancer (Stigbrand and Wahren, 1992). Elevation of serum ALP with hepatic infiltration likely results from compression of small intrahepatic bile ducts. This implies that release of ALP by bile acid fragmentation of membrane and compression of small intrahepatic bile ducts may be the reasons for increased level of ALP in hepatocellular carcinoma.

4.4. Gamma glutamyl transferase (GGT)

In the present study, the serum GGT level was found to be increased in NALC, ALC and HCC categories when compared to the control category. McCullough (2002) and Edoardo *et al.*, (2005) reported that the whole spectrum of liver diseases, regardless of cause may be responsible for altered GGT serum levels. GGT levels may be two to three times greater than the upper reference value in more than 50 % of patients with nonalcoholic fatty liver disease and above the upper reference values in about 30 % of patients with chronic hepatitis C infection. Tumor associated isoenzymes of gamma glutamyl transferase has been reported in HCC (Kojima *et al.*, 1980; Kew *et al.*, 1984). Further more, an increase in GGT levels in patients with chronic liver disease is associated with bile duct damage and fibrosis (Giannini *et al.*, 1999).

GGT is a microsomal enzyme and its activity is found to be induced by several drugs (Rosalki *et al.*, 1971). Edoardo *et al.*, (2005) observed increased serum levels of GGT in alcoholic liver disease and they proposed that, it may be the result of enzyme induction and decreased clearance, and in these patients, GGT serum levels can be markedly altered (>10 times the upper reference values). Increased synthesis of GGT and decreased synthesis of total protein was observed in the HepG2 culture in the presence of alcohol (Moirand *et al.*, 1990). Penn and Worthington (1983) also reported an increased level of hepatic microsomal GGT by the induction of alcohol. In the present study also, the serum level of GGT in ALC category is significantly higher than that of NALC and HCC categories, which implies the increased synthesis of GGT by alcohol.

SERUM ACUTE PHASE PROTEINS

4.5. C- reactive protein

It has been found that the plasma half life of CRP is about 19 hours and is constant in all conditions of health and diseases. So, the sole determinant of circulating CRP concentration is the synthesis rate, which thus directly reflects the intensity of pathological processes stimulating CRP production (Vigushin *et al.*, 1991). In the present study, the CRP level was found to be significantly increased in NALC, ALC and HCC categories when compared to the control group. It is known that serum CRP is elevated in various liver diseases such as acute hepatitis (Atono *et al.*, 1989), cirrhosis (Lee *et al.*, 1989) and hepatocellular carcinoma (Lee *et al.*, 1989; Murakami *et al* 1989). However, those serum levels are not so high as in other inflammatory diseases (Lee *et al.*, 1989). According to early *in vitro* studies, cultured hepatocellular carcinoma cells can produce CRP that is regulated in part by proinflammatory cytokines (Goldman and Liu 1997; Gabay *et al.*, 1995). Patients with hepatocellular carcinoma and cirrhosis had higher CRP levels as reported by

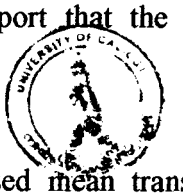
Shiota *et al.*, (1995). Our results also corroborate with this study. In the same study, Shiota *et al.*, (1995) reported that serum hepatocytes growth factor levels were found to be increased in patients with liver disease and serum HGF showed a positive correlation with CRP. Interleukin–6 appears to be the principal regulator of most acute phase proteins (Baumann and Gauldie 1994; Taga and Koshimoto 1992) although other inflammation associated cytokines also contribute to this process.

Huseyin *et al.*, (2006) showed an increase in IL–6 in hepatocellular carcinoma and liver cirrhosis than control, and a highest value was observed in hepatocellular carcinoma. IL–6 is found to be strongly inducing the expression of CRP in human hepatocytes (Streetz *et al.*, 2001; Claire Arnaud *et al.*, 2005). Other cytokines such as IL-1β can also induce CRP production (Pasceri *et al.*, 2000). Cultured hepatoma cells can induce the production of CRP by monocyte factor (Neil and Teh, 1987). All these studies show that CRP is produced by the induction of inflammatory mediators like IL–6, IL–1β and monocyte factors. All these factors are produced in different milieu in cirrhosis and HCC leading to increased production of CRP than normal liver.

4.6. Transferrin

In the present study, serum transferrin level was found to be decreased significantly in all the three diseased categories (NALC, ALC, HCC) when compared to the control category. Transferrin, the main protein, in β fraction, is found to be decreased in liver diseases (Kawai, 1973). Ramadori *et al.*, (1988) observed diminished out put of liver derived proteins, albumin and transferrin, following inflammation and injury. Transferrin is a negative acute phase protein and its synthesis is found to be decreased by interleukins (Thompson *et al.*, 1991). They also showed that insulin is able to inhibit the synthesis of transferrin, which is seen higher in liver diseases with insulin resistance. Hiramatsu *et al.*, (1976) observed increased transferrin levels in chronic and inactive hepatitis but not in

cirrhosis and liver cancer. Shi-Min Luo *et al.*, (2004) reported that the serum prealbumin and transferrin are lower in hepatocellular carcinoma patients with cirrhosis than normal control group. All these studies report that the serum transferrin level is decreasing in liver cirrhosis and liver cancer.



Potter *et al.*, (1985) observed a significantly decreased mean transferrin concentration and decreased synthesis of transferrin in alcoholic liver cirrhotic patients. In the present study highest decrease in serum transferrin was observed in ALC category than HCC and NALC categories. Carbohydrate deficient transferrin is found to be increased in alcoholic hepatitis (Yamauchi *et al.*, 1993). Transferrin is a glycoprotein and it exists in the form of glycosylated isoforms in serum. Specific alterations in the glycosylation of acute phase proteins occur in many pathophysiological states like acute and chronic inflammation and cancer (Hiron *et al* 1992; Feelders *et al.*, 1993). It has been noted that the pattern of change in glycosylation is dependant on the particular state or the particular disease and to some extent on the nature of acute phase proteins. Glycosylated isoforms of transferrin is well studied (Feelders *et al.*, 1993). The biological functions of glycosylated proteins is typically determined by the protein component and, carbohydrate can play a role in molecular stability, solubility, *in vivo* activity, serum half life and in particular can extend the serum half life of protein therapeutics (Elliott *et al.*, 2003). N-glycosylated proteins with high sialic acid content were found to have reduced renal clearance and increased *in vivo* bioactivity (Creus *et al.*, 2001; D' Antonio *et al.*, 1999).

Pradeep and Lekshman (1997) in their animal studies showed marked decrease in the incorporation of labeled sugars into transferrin and a marked decrease in the enzymatic activities of glycosyl transferase and sialyl transferase occurred in rats, chronically administered with ethanol. Alcohol interferes with a number of glycoconjugation reactions, as a result of acetaldehyde inhibition of

hepatic glycotransferases. This inhibition leads to the formation of carbohydrate deficient isoforms of transferrin (Fleming *et al.*, 2004; Golka and Weise, 2004; Wuyts and Delanghe, 2003). Consumption of large amount of alcohol is found to be causing decreased sialylation of transferrin (Vesterberg *et al.*, 1984). It is also proposed that there is reduced activity of liver glycoprotein glycosyltransferase (Stibler and Borg, 1991; Ghosh *et al.*, 1993) as well as increased hepatic and plasma activities of sialidase (Ghosh *et al.*, 1993) associated with alcohol consumption. Stibler and Borg (1986) reported that chronic alcohol consumption reduces the number of carbohydrate moieties attached to serum transferrin and producing carbohydrate deficient transferrin. It has been found that the carbohydrate deficient transferrin occur in elevated levels in the blood of alcoholics (Stibler, 1991). The half life of carbohydrate deficient transferrin in serum is found to be very low. So, in ALC category the carbohydrate deficient transferrin is easily cleared from the serum leading to a decrease in transferrin than in NALC and HCC categories.

4.7. Ferritin

Isolated and non specific increase in serum ferritin levels are frequently found in the absence of iron over load and are associated with inflammation, liver necrosis and alcohol abuse (Bacon *et al.*, 1994). In our study, serum ferritin was found to be increased significantly in HCC and ALC categories than in NALC and control categories. Bacon *et al.*, (1994) also reported abnormal serum ferritin level in 58 % of patients with non alcoholic steatohepatitis. In our study also there is a slight increase in serum ferritin levels in non alcoholic liver cirrhosis, but that was not significantly different from the control category.

Many studies have shown that serum ferritin level is increased in liver cirrhosis and HCC. The study of Caroline *et al.*, (2004) showed a significant increase in ferritin in liver cirrhosis than the control category. Rat liver and spleen ferritin synthesis is elevated 3–4 folds six hours after the onset of an experimentally

induced inflammatory response (Konijn *et al.*, 1977; Campbell *et al.*, 1989). Bell *et al.*, (1994) found that serum ferritin is elevated in alcohol abusing patients with alcoholic liver disease than in patients with chronic liver disease. Serum ferritin and transferrin saturation can be increased in alcoholic liver disease (Brissot *et al.*, 1981; Chapman *et al.*, 1982). Many studies showed an elevated value of serum ferritin in alcoholic liver disease (Loreal *et al.*, 1992; Bufler *et al*,. 1995; Verhasselt *et al.*, 1997; Sugawara 1998). In a HepG2 culture study, it was reported that there is an increased synthesis of ferritin and decreased total protein in the presence of alcohol (Moirand *et al.*, 1990).

It has been found that IL–1β stimulates ferritin production by liver hepatoma cells (HepG2) because plasma iron level characteristically falls during acute phase response (Bazil *et al.*, 1991). This reduction in iron level results from an increase in liver ferritin synthesis by IL-1β as demonstrated in a rat model by Konijn and Hershko (1981). Jack *et al.*, (1990) reported that human hepatoma cells when stimulated with IL-1β, do exhibit a marked increase in the steady state level of ferritin shells and which can be measured by protein staining on non denaturing gels. They also suggest that ferritin protein accumulate in these cells rather than being degraded. There is also evidence that increased rat liver ferritin synthesis is controlled in the level of transcription during the acute phase response (Konijn 1981; Campbell *et al.*, 1989). IL–1β is high in HCC than in cirrhosis. Increased level of IL–1β may be enhancing the synthesis of ferritin in HCC. In alcoholic liver cirrhosis, alcohol induces the synthesis of ferritin and release of ferritin from hepatocytes due to tissue necrosis as well as the induction of IL-1β for the synthesis of ferritin in hepatocytes. The over all effects may be contributing to the increased level of serum ferritin in ALC category than HCC category in the present study.

4.8. Ceruloplasmin

In our study, the ceruloplasmin level was found to be increased significantly in HCC category when compared to control as well as NALC and ALC categories. Ceruloplasmin is a member of acute phase protein family and its level is found to be increased during inflammation as well as in various malignancies (Gitlin, 1988; Ramadori *et al.*, 1988). Diehl (1999) reported an increased ceruloplasmin concentration in the serum of hepatocellular carcinoma patients when compared to chronic hepatitis patients. Fey *et al.*, (1994) reported that the elevation of ceruloplasmin during acute phase response is usually less than two fold. In the present study also there is a significantly increased level, but less than two fold increase of ceruloplasmin in HCC category when compared to control. Inflammatory cytokines, including interleukins locally produced in the liver cells are inducers of ceruloplasmin synthesis in cirrhotic patients (Ramadori *et al.*, 1988). Hepatocellular hypoxia was demonstrated in experimental cirrhosis (Corpechot *et al.*, 2002), which could induce an increased synthesis of ceruloplasmin mRNA (Mukhopadhyay *et al.*, 2000). In the present study also some cases in NALC and ALC categories showed a slight increase in ceruloplasmin level than the control category, though there was no statistical significance.

Earlier studies reported a modest increase in ceruloplasmin concentration in the serum of hepatocellular carcinoma patients in comparison with chronic hepatitis patients (Casaril *et al.*, 1989). Our studies reveal that the serum ceruloplasmin concentration of HCC category showed a significant increase when compared to NALC and ALC categories. The study of Dominique *et al.*, (2001) showed that ceruloplasmin accumulated to extremely high levels in the serum of the transgenic mice developing hepatocellular carcinoma and they proposed that the accumulation of ceruloplasmin in the serum is due to the increased expression of ceruloplasmin genes as well as an increase in the mRNA stability. Ross, (1995) revealed that the half life of ceruloplasmin mRNA is increased in the transgenic mice. It has been

proposed that a longer mRNA half life may increase the rate of translation, leading to this elevated condition (Ross, 1995).

It is also noted that the amount of plasma ceruloplasmin is 10 times higher in humans than in mice, and the variation in the normal ceruloplasmin values within the human population is large (250 – 630 mg/L) (Ross, 1995). The results in our study also showed the same pattern. The mean value of ceruloplasmin is near to the upper reference limit, and about 11 out of 38 cases showed ceruloplasmin values above the upper reference limit. It is reported that moderately increased levels of ceruloplasmin is observed in human hepatocellular carcinoma, and is a prognostic value for elevated plasma ceruloplasmin concentration corresponding to more rapidly progressing tumor (Casaril *et al.*, 1989; Knekt *et al.*, 1992; Senra *et al.*, 1997). Dominique *et al.*, (2001) also reported that ceruloplasmin concentration increases with tumor mass. This indicates that the increased level of ceruloplasmin, above the reference limit, in some cases in HCC category, in our study, may be due to the increased tumor growth.

Ceruloplasmin is a glycoprotein and its half life in the plasma is determined by the extent of the sialylation of its N-glycan chains (Morell *et al.*, 1971). It has been found that transgenic mice express in their liver much higher amount of the galactoside and sialyl transferase that transfer sialic acid specifically to N–glycans (Pousset *et al.*, 1997). N-linked glycosylation can increase *in vivo* potency of molecule through elevating its half life (Angus *et al.*, 2005). All these studies showed that the level of ceruloplasmin is increased in HCC probably due to increased mRNA synthesis, increased mRNA half life and increased half life of ceruloplasmin.

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Chapter 5

CONCLUSION

Control compared with NALC

When compared to the control category, NALC category showed a significant increase at 1% level in the mean values of serum bilirubin, AsT, AlT, GGT, CRP and AST/AlT ratio. The NALC category showed a statistically significant decrease in serum albumin level. The serum levels of ALP, ferritin, ceruloplasmin and transferrin values were not statistically significant when compared to the control group.

Control compared with ALC

The ALC category showed a statistically significant increase in the serum values of bilirubin, AsT, AlT, GGT, CRP, ferritin and AsT/AlT ratio and there was a significant decrease at 1 % level in the serum values of albumin and transferrin when compared to the control category. The serum values of ALP and ceruloplasmin in the ALC category were not statistically significant when compared to the control category.

Control compared with HCC

The serum values of bilirubin, AsT, AlT, ALP, GGT, CRP, ferritin, ceruloplasmin and AsT/AlT ratio of HCC category showed a statistically significant increase when compared to the control category. When compared to the control category, HCC category showed a statistically significant decrease in the serum albumin and transferrin values.

NALC compared with ALC

When compared to NALC category, ALC category showed a significant increase in the serum values of bilirubin, AsT, GGT, ferritin and AsT/AlT ratio. There was a significant decrease in the serum transferrin level. There was a change in the serum values of AlT, ALP, CRP, ceruloplasmin and albumin in ALC

category, when compared to NALC category and these values were not statistically significant.

NALC compared with HCC

When NALC and HCC categories were compared, the serum levels of bilirubin, ALP, ferritin and ceruloplasmin of HCC category showed a statistically significant increase and albumin showed a statistically significant decrease. The serum levels of AsT, ALT, GGT, CRP and AsT/AlT ratio of NALC and HCC categories showed no statistical significance.

ALC compared with HCC

The serum levels of AsT, ALP, GGT, ferritin, ceruloplasmin and AsT/AlT ratio of ALC category was statistically significant when compared to the HCC category. The serum levels of bilirubin, albumin, AlT, CRP and transferrin of ALC category showed no statistical significance when compared to HCC category.

Categories showing highest and lowest values

Among the different disease categories, ALC showed a highest increase in the serum values of bilirubin, AsT, AlT, GGT, ferritin and AsT/AlT ratio and a highest decrease in the serum values of transferrin, HCC showed a highest increase in the serum values of ALP, CRP and ceruloplasmin and a highest decrease in the serum albumin value. The NALC category showed lowest values in serum bilirubin, AsT, ALP, GGT, CRP and ferritin and lowest decrease in the serum value of transferrin and Albumin.

Order of increase of parameters in different categories

When various serum parameters of different categories were compared, the increasing order of NALC<HCC<ALC was found in the values of bilirubin, ALT, GGT, AST and ferritin, increasing order of NALC < ALC <HCC was found in the

values of CRP and ALP, increasing order of ALC < NALC < HCC was observed in the ceruloplasmin value and an increasing order of HCC < NALC < ALC was observed in AsT/AlT ratio.

Order of decrease of parameters in different categories

A decreasing order of NALC > ALC > HCC for albumin and a decreasing order of NALC > HCC > ALC for transferrin were observed when various serum parameters of different categories were compared.

Serum enzyme pattern in different categories

Among the serum enzymes, AsT, AlT, and GGT values were very high in ALC category than NALC and HCC categories, while ALP value was found to be very high in HCC than NALC and ALC categories.

Serum acute phase proteins pattern in different categories

When the values of acute phase proteins in different categories were compared, the CRP and ceruloplasmin values were found to be higher in HCC category than NALC and ALC categories, while ferritin value of ALC category was found to be higher than NALC and HCC categories. The value of transferrin was found to be lower in ALC than NALC and HCC categories.

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