Training Report

BIOCHEMICAL AND IMMUNOASSAY FOR THE DIAGNOSIS OF HEPATITIS C VIRUS

Internship Training Report

Submitted to

Lovely Professional University, Punjab

in partial fulfillment of the requirements

For the degree of

Master of Science in Clinical Biochemistry

Submitted by:
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CERTIFICATE

This is to certify that Mr./*Ms. Simarpreet kaur* bearing **Registration Number** *11401051* has completed his/her Master of Science in Clinical Biochemistry internship under our guidance and supervision. This report is record of the candidate own work carried out by him/her under my supervision. I certify that the matter embodied in this report is original and has been not submitted anywhere for the reward of any other degree.

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DECLARATION

I hereby declare that the work embodied in this internship report was carried by me under the supervision of Dr Ekta Chitkara (Internal supervisor), Associate professor of Paramedical science, Lovely Professional University and Dr Shveta Goel (External supervisor), Pathologist, Mannat Hospital. This work has not been submitted in part or in full in any other university for any degree or diploma.

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ABSTRACT

Hepatitis C virus infection poses a growing challenge to health care systems. Although Chronic HCV infection begins as an asymptomatic condition with few short term effects, it can progress to cirrhosis, hepatic decompensation, hepatocellular carcinoma (HCC), and death. Progression of fibrosis can be accelerated by factors such as older age, duration of HCV infection, sex, and alcohol intake.

Screening for hepatitis C leads to the appropriate evaluation and treatment of individuals chronically infected with th hepatitis C virus and prevents the progression of liver disease to cirrhosis, hepatocellular carcinoma, and associated morbidity and mortality. Screening for Hepatitis C virus is also cost effective. Diagnosis is by blood testing to look for either antibodies to the virus or its RNA. Testing us recommended in all people who are at risk .

The assay has better sensitivity and permits the detection of HCV infection earlier than the HCV antibody screening tests and an average of only two days later than quantitative HCV-RNA detection in individual specimens. The performance of the assay correlates well with those of molecular HCV RNA detection methods, but the lower level of detection is significantly higher.

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To all of these individuals I owe many thanks for their insight and unlimited support.

Simarpreet Kaur.
Signature of Student

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LIST OF ABBREVIATION

1. HBV	Hepatitis B virus
2. HCV	Hepatitis C virus
3. HAV	Hepatitis A virus
4. RNA	Ribonucleic acid
5. HCC	Heptocelluar Carcinoma
6. ALD	Alcoholic liver disease
7. NASH	Non alcoholic steatohepatitis
8. IRES	Internal ribosome entry site
9. NS	Non structural
10. WHO	World health organization
11. GGT	Gamma-glutamyl transferase
12. ALT	Alanine amino transferase
13. AST	Aspartate amino transferase
14. ALP	Alkaline phosphate
15. EIA	Enzyme immunoassay
16. CHC	Chronic hepatitis C
17. ELISA	Enzyme linked immune assay
18. RIBA	Recombinant immunoblot assay
19. SD	Standard deviation

CHAPTER 1

INTRODUCTION

Viral hepatitis is a systemic disease primarily involving the liver and is caused by many viral agents which are etiologically, immunologically and epidemiologically distinct. Hepatitis C was first recognized in early 1970, after the discovery of hepatitis A virus (HAV) and hepatitis B virus (HBV) (1). It was noticed at that time that most cases of transfusion-associated hepatitis were not caused by either these viruses, this virus was called "non A non B". In 1989, the responsible virus for most transfusion-associated non A-non B hepatitis was identified through molecular biological techniques and Hepatitis C virus (HCV) was cloned. Hepatitis C is an enveloped virus, with a diameter of about 50-60 nm, positive sense, single stranded RNA virus. The HCV genotype is an intrinsic characteristic of the transmitted HCV strains and does not change during the course of the infection. HCV genotypes from six clades or types (number from 1-6) and are themselves subdivided into number of subclades or subtypes identified by lower case letter (1a, 1b, 1c etc). Probably as many as 70%-90% of infected people fail to clear the virus during the acute phase of the disease and become chronic carrier (2). Chronic hepatitis C is highly heterogeneous and many patients present with mild form of liver disease. The assay has better sensitivity and permits the detection of HCV infection earlier than the HCV antibody screening tests and an average of only two days later than quantitative HCV-RNA detection in individual specimens. The performance of the assay correlates well with those of molecular HCVRNA detection methods, but the lower level of detection (20,000 IU/ml) is significantly higher (3).

1.1 NORMAL LIVER

The liver is the largest organ of the human body weighs approximately 1500 g, and is located in the upper right corner of the abdomen. The organ is closely associated with the small intestine, processing the nutrient-enriched venous blood that leaves the digestive tract (4). The liver performs over 500 metabolic functions, resulting in synthesis of products that are released into the blood stream (e.g. glucose derived from glycogenesis, plasma proteins, clotting factors and urea), or that are excreted to the intestinal tract (bile). Also, several products are stored in liver parenchyma (e.g. glycogen, fat and fat soluble vitamins).

There are two anatomical lobes; the rights being about six times the size of the left lesser segments of the right lobe are the caudate lobe on the posterior surface and the quadrate lobe

on the inferior surface. The right and left lobes are separated anteriorly by a fold of peritoneum called the falciform ligament posteriorly by the fissure for the ligamentum venosum and inferiorly by the fissure for the ligamentum teres (4).

Liver cells can be classified into the following three groups:

1) Parenchymal cells, which comprise hepatocytes and the bile duct epithelia,
2) Sinusoidal cells, which comprise hepatic sinusoidal endothelial and Kupffer cells (hepatic microphage).
3) Perisinusoidal cells, which consist of hepatic stellate cells and pit cells. Hepatocytes account for 60% of the adult liver cell population and represent 78% of the tissue volume (5).

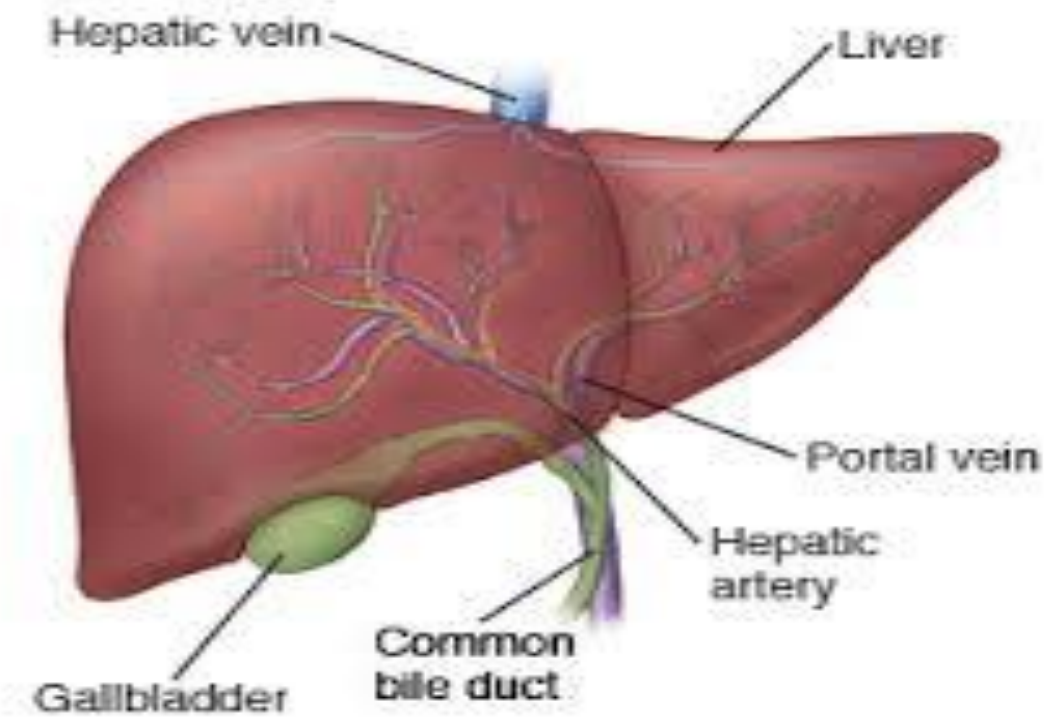

Figure 1: Showing Anterior view of the liver

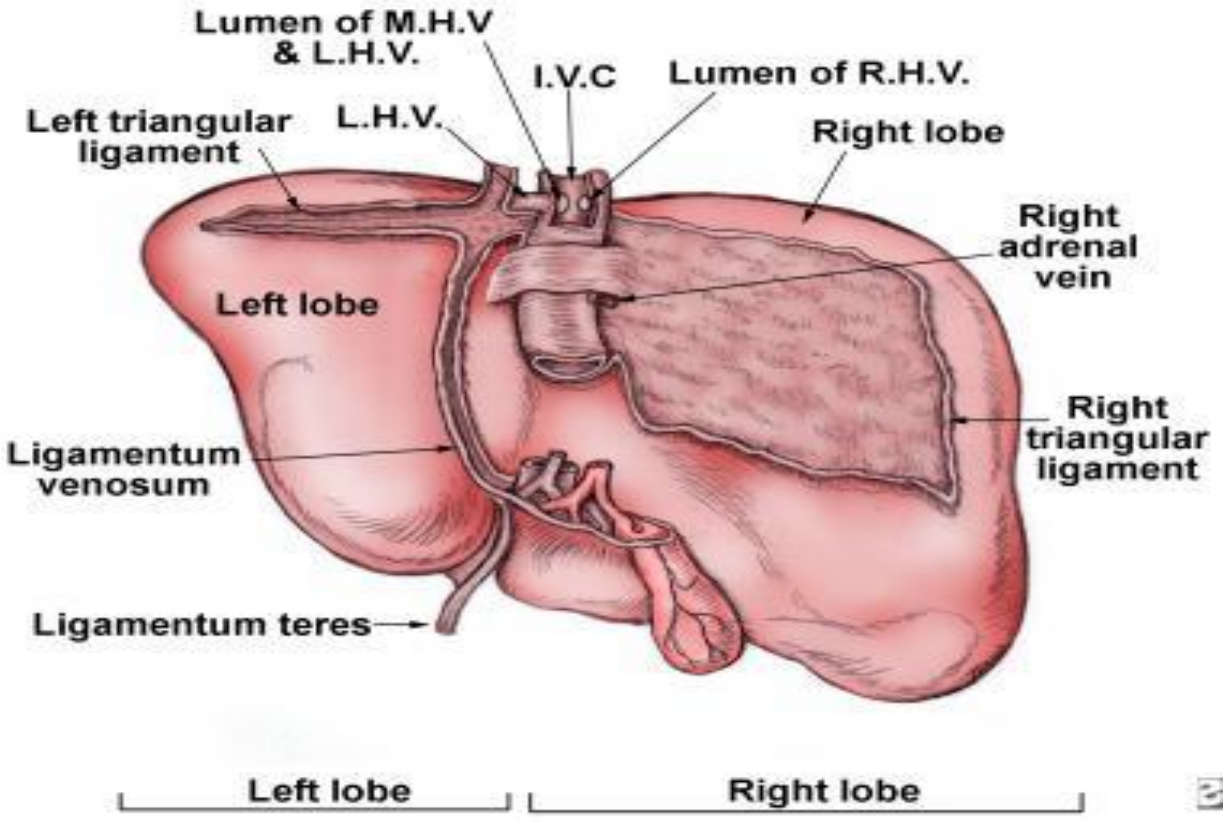

Figure 2: Showing Posterior view of the liver

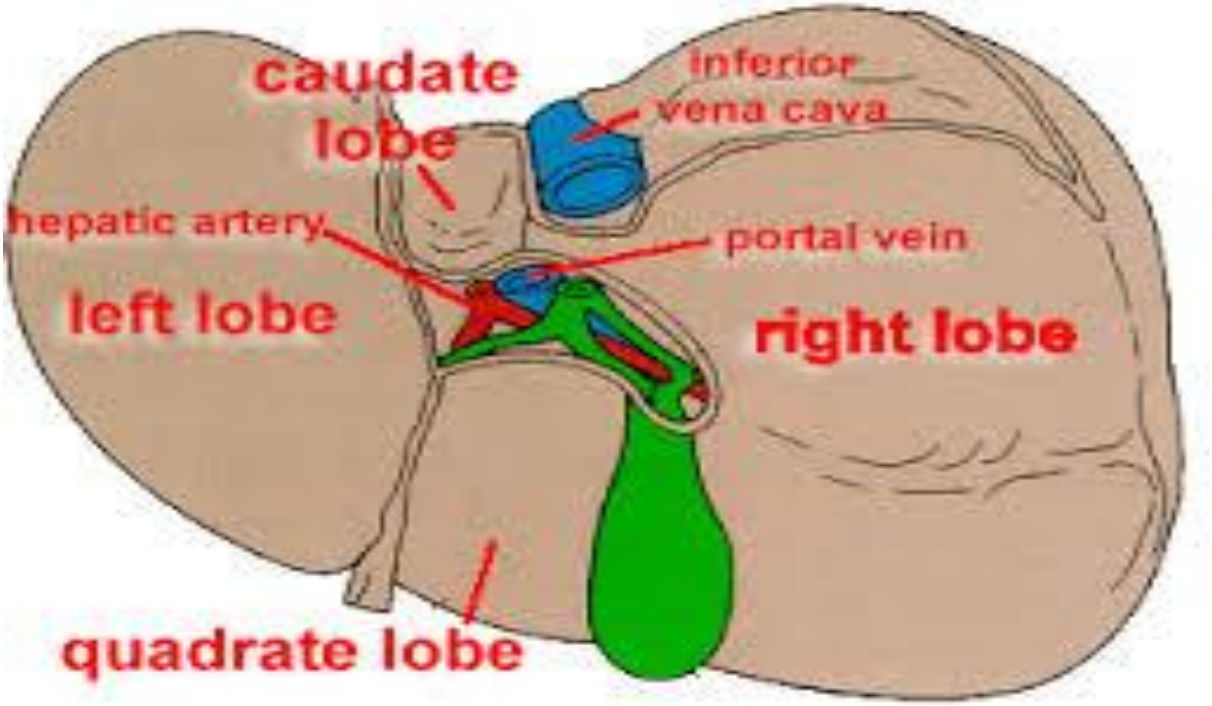

Figure 3: Showing Inferior view of the liver

Hepatocytes are polarized epithelial cells. Their plasma membranes have three distinct domains:

(1) The sinusoidal surface (≈37% of the cell surface) that comes in direct contact with plasma through the fenestrate of the specialized hepatic sinusoidal endothelial cells.
(2) The canalicular surface (≈13% of the cell surface) that encloses the bile canaliculus.
(3) Contiguous surfaces.

The sinusoidal and canalicular surfaces contain microvilli, which greatly extend the surface area of these domains. The space between the endothelia and the sinusoidal villi is termed space of Disse. (6)

1.2LIVER FUNCTIONS

The normal liver has a huge reserve functional capacity. When the liver is normal, about 80% of it can be removed without compromising function, the liver has synthetic, excretory, and metabolic functions. (7)

Synthetic functions

The liver is the source of plasma albumin, many plasma globulins, including α1-antitrypsin and many proteins coagulation cascade. (8)

Excretory functions

Many substances are excreted by the liver in bile. The main component of bile is bilirubin, cholesterol, urobilinogen, and bile acids are also present in bile. (9)

Metabolic functions

The liver plays a central role in the metabolism of fat, carbohydrates, protein and in detoxification:

a) Fat metabolism

Free fatty acids from adipose tissue and medium or short chain fatty acids absorbed in the intestine brought to the liver. Triglyceride, cholesterol and phospholipids are synthesized in the liver from the fatty acids and complexed with specific lipid acceptor proteins to form very low density lipoproteins that enter the plasma. The liver also metabolizes intermediate and low density lipoprotein. (10-11)

b) Carbohydrate metabolism

The liver is the main source of plasma glucose. Following a meal, glucose is derived from intestinal absorption in the fasting state, glucose is derived from glycogenolysis and gluconeogenesis in the liver. The liver is the main body storage site for glycogen.

When there is a glucose deficiency, the liver metabolizes fatty acids to form ketone bodies, which represent an alternative energy source for many tissues. (12)

c) Protein metabolism

In addition to its synthetic function, the liver is the central organ in protein catabolism and synthesis of urea. Urea is secreted by the liver into the plasma for excretion in the kidney. (13)

d) Detoxification

The liver plays a vital role in detoxifying noxious nitrogenous compounds derived from the intestine and many drugs and chemicals. (14)

1.3 LIVER DISEASES

Liver diseases affect the normal functions of the liver. Abnormalities in the liver functions, however, are usually not apparent in most individuals with chronic liver disease until the disease is rather advanced. (15) The diseases that occur in the liver are infectious (e.g. viral hepatitis), toxic (e.g. alcohol-related diseases), genetic (e.g. hemochromatosis), immune (e.g. autoimmune hepatitis primary biliary cirrhosis), and neoplastic (e.g. hepatocellular carcinoma). (16)

a) Liver hepatitis

Hepatitis is inflammation of the liver. Viral infection is responsible for around half of all cases of acute hepatitis. The term is generally used to refer to the diseases caused by the hepatropic viruses including the diseases hepatitis A–E, and disease due to cytomegalovirus, Epstein-Barr virus, adenovirus, rarely herpes simplex virus and others. (17) Among the hepatitis viruses, only hepatitis B virus and hepatitis C virus are able to persist in the host and cause chronic hepatitis. (18) Hepatitis C virus is also a major public health problem. (19)

b) Acute hepatitis

Descriptions are largely based on findings in transfused patients where the time of infection is certain. Clinical presentation of disease after other modes of transmission, such as intravenous drug addiction. The incubation period is about 7-8 weeks (range 2- 26 weeks). The symptoms resemble those of other forms of viral hepatitis. Serum

Hepatitis C virus ribonucleic acid (RNA) becomes positive 1-2 weeks after infection. At 7-8 weeks, serum alanine aminotransferase is moderately increased to about 15 times the upper limit of normal. Clinical diagnosis is rarely made and this depends on viral markers. (20)

c) Chronic hepatitis

Chronic hepatic injury is a relatively common disorder with minimal symptoms but long-term risk of significant morbidity and mortality. It is defined pathologically by ongoing hepatic necrosis and inflammation of the liver, often accompanied by fibrosis. Chronic hepatic injury may progress to cirrhosis (15-20 % in the case of chronic hepatitis C virus) and predisposes to hepatocellular carcinoma. Most commonly, it is the result of chronic viral infection. (21)

d) Drug and alcohol related disorders

Toxins such as alcohol, drugs or poisons can cause hepatitis directly (by damaging liver tissue) or indirectly (by reducing defenses or stimulating an autoimmune response), but the exact mechanism is not always clears. (22)

e) Liver cirrhosis

Chronic hepatitis C leads to cirrhosis in 20 to 30%. Hepatocellular carcinoma can develop in 1 to 5%. This natural course is modified by several factors including age, sex and alcohol. This last one is an important risk factor for fibrosis, cirrhosis and hepatocellular carcinoma. Data about high alcohol consumption show an increased risk whereas the risk associated with light to moderate consumption of alcohol remains unclear. (23)

f) Hepato-cellular carcinoma (HCC)

Alcoholic hepatitis and nonalcoholic steatohepatitis (NASH) show different clinical features with similar liver histology, but both disorders may progress to cirrhosis and HCC. HCC arising in alcoholic liver disease (ALD) or NASH, without hepatitis B or C virus infection, has been a rare observation and there are no studies comparing the characteristics of alcoholic liver disease and nonalcoholic steatohepatitis patients with HCC. (24)

1.4HEPATITIS C VIRUS

Hepatitis C virus is a small (~55 to 65nm), spherical, enveloped, hepatotropic RNA virus that causes acute and chronic hepatitis. Hepatitis C virus (HCV) is a large health care burden to the world. (25) HCV is the most common blood born infection and is the leading cause of chronic liver disease in the USA. HCV is both a hepatotropic and a lymphotropic virus; and chronic infection could cause, chronic hepatitis, cirrhosis.The clinical course of chronic HCV is highly variable ranging from mild hepatitis (inflammation of the liver), fibrosis (scaring of the liver), cirrhosis (end-stage fibrosis) to HCC. (26)

1.5HISTORY OF HEPATITIS

In 1960, Baruch Blumberg was researching genetic links to disease susceptibility. During this time, he accidentally discovered the hepatitis B (HBV) virus in the blood sample of an Australian Aborigine. This discovery led to the development of a test to screen people for HBV. This also led to an effective vaccine for the disease. In 1976, Blumberg was awarded the Nobel Prize for his work. (27) In 1989, The Centers for Disease Control and Prevention and Chiron came together to identify the hepatitis C (HCV) virus. There isn't a vaccine for HCV at this time.

1.6 MOLECULAR VIROLOGY OF HCV

Genomic organization of HCV

HCV is a small enveloped virus composed of a host cell derived lipid membrane that carries the envelope glycoproteins and a nucleocapsid that harbors the single strand RNA genome of positive sense polarity which acts as template in replication and translation processes. HCV is a positive strand RNA virus that has been classified as the genus Hepacivirus in the Flaviviridae family. The HCV genome is an uncapped, linear molecule with a length of 9600 nucleotides coding for a polypeptide with a length of about 3,000 amino acids. (28) it carries a long open reading frame that is flanked at the 5` and 3` ends by short highly structured non translated regions(NTRs). The 5` NTR has a length of about 341 nucleotides and contains an internal ribosome entry site (IRES) required for translation of the HCV genome. The RNA element binds the 40 structure ribosomal subunit in the absence of other translation initiation factors in a way that the initiation codon is placed in the immediate vicinity of the P site. (29) Part of the internal ribosome entry sit (domin II) overlaps with RNA signals essential for viral

replication arguing for a possible role of domin II in regulating a translation RNA replication switch . (30) The 3` NTR has a tripartite structure composed of an 41 nucleotides long variable region downstream of the HCV coding sequence, a poly-(U)-polypyrimidine tract of heterogeneous length, and highly conserved 98 –nucleotide sequence designated X-tail. Expression of the viral proteins from the monocirstronic genome is primarily achieved by production of a polyprotein that is proteolytically cleaved into the structural proteins(core, envelope proteins E1 and E2), the hydrophobic peptide p7, and the non structural(NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Processing of the core to p7 region is mediated by host cell signalases, and in the case of the core protein, in addition by signal peptide peptidase. All remaining cleavages are carried out by two viral proteases: the NS2/3 protease mediating cleavage between NS2 and NS3 and the NS3 serine type protease that responsible for processing at all other sites in the NS polyprotein region. (31) Alternative forms of the core protein generated by internal initiation of translation of an alternative reading frame or by ribosomal frameshifting have been reported.

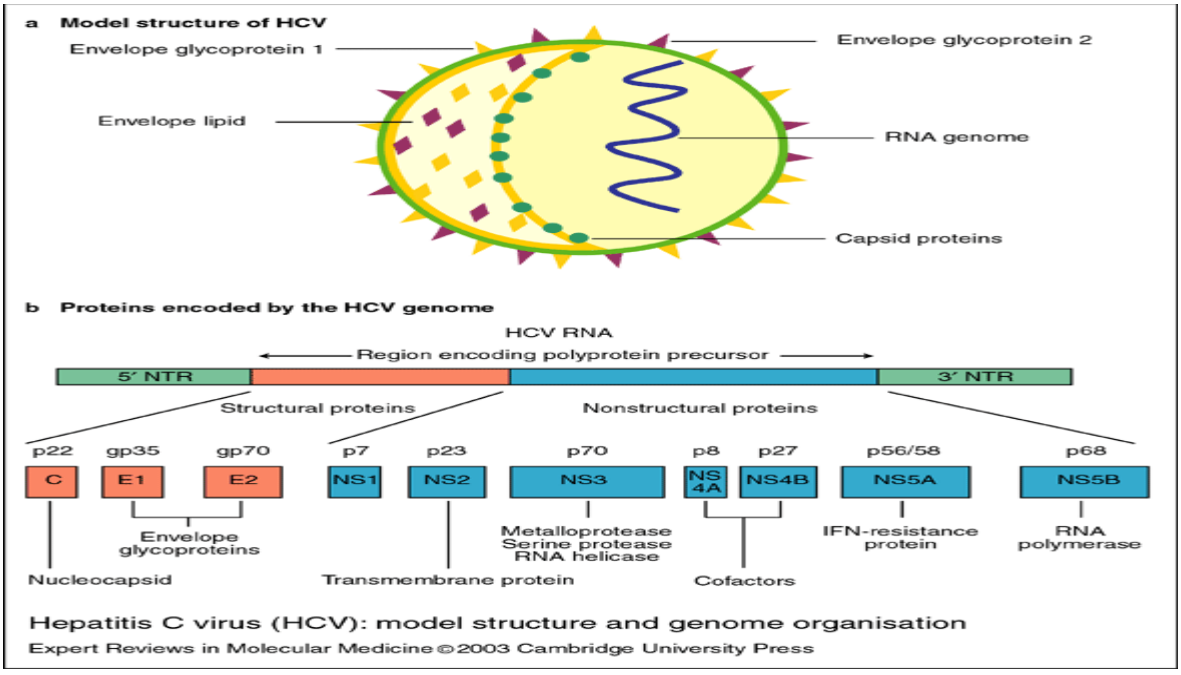

Figure 4: Structure and genome organization of HCV virus

1.7 Life Cycle of HCV

After the landmark report of the first HCV DNA clones in 1989, many expected HCV molecular virology to quickly advance to a state rivaling that of other positive-strand RNA viruses. (32) More than 15 years later, this predication is only beginning to be realized. Significant advances in understanding the key steps of the HCV life cycle have been made in recent years, although many steps remain enigmatic.

a) Extracellular HCV virions interact with receptor molecules at the cell surface
b) And undergo receptor-mediated endocytosis into a low-pH vesicle.
c) Following HCV glycoprotein-mediated membrane fusion, the viral RNA is released into the cytoplasm.
d) The genomic RNA is translated to generate a single large polyprotein that is processed into the 10 mature HCV proteins in association with a virus derived ER-like membrane structure termed the membranous web.
e) The mature HCV proteins replicate the RNA genome via a minus-strand replicative intermediate to produce progeny RNA.
f) A portion of this newly synthesized RNA is packaged into nucleocapsids and associated with the HCV glycoproteins, leading to budding into the ER .
g) Virions follow the cellular secretory pathway and, during this transit, maturation of particles occurs.
h) Mature virions are released from the cell, completing the life cycle. (33)

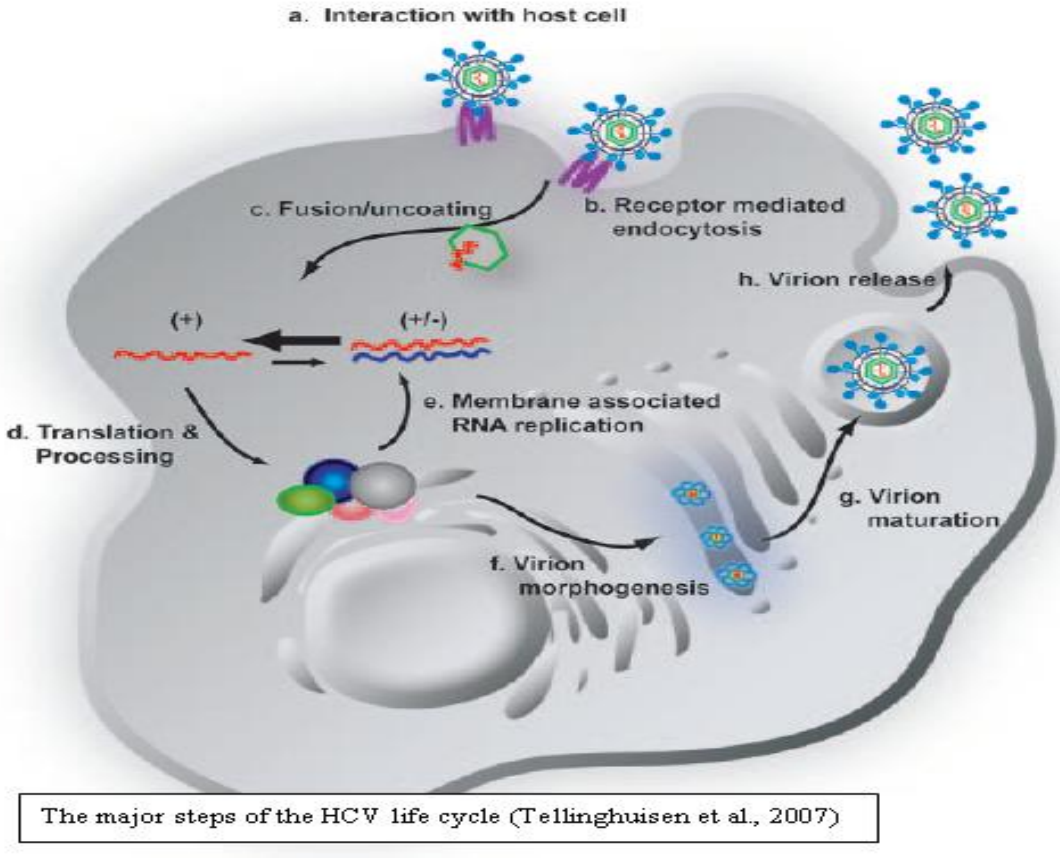

Figure 5: Schematic diagram of the HCV life cycle

1.8 Epidemiology of HCV

HCV is recognized as a major threat to the global public health. Globally, HCV is estimated to infect over 170 million individuals (about 3% of the world‘s population). World health organization (WHO) published their first estimate of global prevalence of HCV. The WHO regions where prevalence was reported to be high are Africa, the Eastern Mediterranean, South-East Asia and the Western Pacific. Lower prevalence was found in countries in North America and Europe. (33) Prevalence rates across the world have changed as well with more countries aware of transfusion related HCV and more and more evidence supporting intravenous drug use as the leading risk factor of spread of the virus. (34)

In Europe, general prevalence of HCV is about 1% but varies among the different country. (35) In Northern Italy, prevalence of HCV antibody was 3.2 %. The prevalence of HCV was estimated to be 1.3%, very similar to the French general population. (36) Very low prevalence has been reported in UK and Scandinavian 0.04-0.09% , and also in some areas of Japan. Low prevalence ranging from 0.15 to 0.5% in US, Western Europe. (37)

1.9 Transmission of HCV

The transmission of and screening for HCV infection varies considerably throughout the world; differences between resource-poor and resource-rich countries are particularly pronounced. The primary mode of HCV transmission is exposure to infected human blood via intravenous drug use or unscreened transfusions. Nosocomial HCV transmission during dialysis, colonoscopy, and surgery has also been reported. (38) The modes of transmission of HCV infection can be divided into percutaneous (blood transfusion and needle stick inoculation), and non percutaneous (sexual contact, perinatal exposure). The latter group may represent occult percutaneneous exposure. Over all, blood transfusion from unscreened donors. Perinatal and sexual transmission of the virus is inefficient. Indeed, in initial studies, HCV was shown to be the etiologic agent in more than 86% of cases of post-transfusional non A, non B hepatitis. (39)

1.10 Diagnosis of HCV infection

The molecular or virologic assays detect specific viral nucleic acid sequences (HCV-RNA), which indicate persistence of the virus. (40) Whereas serologic assays are typically used for screening and first line diagnosis; virologic assays are needed to confirm active infection or to monitor the effects of the therapy.

1. **Liver enzymes and Liver function testes**

   An increment of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) in human serum indicates an abnormal symptom of the liver. (41) The diagnosis of chronic hepatitis C infection often is suggested by abnormalities in ALT levels and is established by enzyme immunoassay (EIA) followed by confirmatory determination of HCV RNA. (42) AST can be very high in acute hepatitis and drop to normal or slightly elevated in chronic hepatitis. ALT is very useful for monitoring the effectiveness of antiviral drug treatment for CHC. The response to treatment is judged in terms of ALT normalization during and after treatment. (43) Although it is generally true that patients with an active and progressive hepatitis will have elevated liver enzymes, it is not true that patients with normal liver enzymes will have no liver damage. (44)

2. **Serologic assays**

   Secretion of antigen-specific and non-specific immunoglobulins can be detected by enzyme-linked immunosorbent assay (ELISA). A number of tests are available for detecting the presence of anti-HCV. (45) The most commonly used tests are the

enzyme immunoassay (EIA) and ELISA, which is a type of EIA. These tests are inexpensive, rapid, easy to perform and widely available. Early versions were plagued by frequent false-positive reactions, but the current "third-generation" assays are 99% specific and 99% sensitive in immune competent individuals. (46) Patients who react with two or more HCV antigens by recombinant immunoblot assay (RIBA) are considered to have tested positive, while those who react to one antigen have indeterminate results. The RIBA and ELISA share the same sensitivity. (47) Compared to ELISA, RIBA has a higher specificity. The second and third generation immunoblot tests are based on the detection of anti-HCV antibodies with structural and non structural viral antigens coated as parallel bands on nitrocellulose strips. Immunoblot tests are useless as confirmatory assays in routine diagnosis of HCV infection in clinical laboratories, but can resolve false-positive results in the blood donor screening context. (48)

3. **Virologic assays**

Quantitative assays: Quantitative measurements of serum HCV RNA are becoming increasingly important in the management of HCV-infected patients. (49) Quantitative HCV RNA testing provides prognostic information regarding likelihood of treatment response and plays an important role in monitoring the antiviral response to treatment. (50)

Qualitative assays: Qualitative molecular tests are useful for confirmation of positive screening tests**.** Qualitative nucleic acid tests have low limits of detection (<50 IU HCV RNA/ML) and are used for confirmation of HCV infection and for screening blood donations**.** Qualitative tests are more sensitive than quantitative ones, because they detect very small quantities of viral RNA. (51)

CHAPTER 2

REVIEW OF LITERATURE

Higuchi M et al; (2002)

Conducted study which shows that HCV infects an estimated 170 millions persons worldwide and 2 million persons in Japan. HCV is a major cause of chronic liver diseases, especially Heptocarcinogensis and the number of patient with HCV related Hepatocellular carcinoma is increased worldwide as well as in Japan.

Suresh D. Sharma et al; (2010)

Conducted study on molecular biology of Hepatitis C virus and on Structural proteins. This study shows morphologic and genomic organization of Hepatitis C Virus. The non structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B are thought to be to be required for replication of the viral genome. The non structural protein, p7, can form ion channels, required for the production of infectious virus particles (52).

Sonia Sharma et al; (2013)

Conducted study which shows that Hepatitis B and C virus infections had increased serum transaminases with histological evidences of liver damage. However, there was no correlation between the histological grades and the biochemical parameters (53).

Muhammad Shahid et al; (2015)

Conducted study which shows that level of ALT, AST, ALP and billirubin had significant relation with the liver fibrosis stage and the inflammatory grade in genotype 3a. This study suggests that AST, ALP and ALT may correlate with liver damage. Serum HCV RNA titers was correlated with AST,ALP and total billirubin, levels of ALT,AST ALP and billirubin had significant relation with the liver fibrosis stage and the inflammatory grade in genotype 3a. Hence, this study suggests that AST, ALP, and ALT may correlate with liver damage (54).

Klaus Langohr et al; (2008)

Conducted study which shows that routinaly marker of liver diseases or liver inflammation to analyze the correlates of AST, ALT and GGT elevations. This study also shows that elevation of AST and ALT are more frequent in men compared to women from general population.

Elevations of AST and ALT are more frequent in men compared to women from general population. In addition, gender has been associated with the prognosis of liver disease in patients with chronic hepatitis C (55).

Isabelle Desombere et al; (2005)

In this study they measured the HCV RNA levels in sera from 12 chronic HCV patients using two commercial quantitative assays: the HCV Monitor 2.0 assay with automated HCV RNA extraction (Ampliprep) and the HCV bDNA 3.0 assays, which is a direct technology that does not use HCV RNA extraction. In the present study they compared the quantitative results obtained by both assays. Since comparison of the HCV RNA data from 344 patient specimens resulted in only 5% discrepant results, we can conclude that there is an overall concordance between both systems. Comparison of the HCV RNA titers within the dynamic ranges of both assays revealed a good correlation (r 2 0.86), and they conclude that both assays perform equally well in determining HCV viral loads (56).

Chi Hoon Lee et al; (2013)

Conducted a study in which 490 patients were included. The result of HCV RNA test is was positive and negative in 228 and 262 patients, respectively. This study was conducted to identify the predictive factors associated with positive results of an HCV RNA test in the case of positive screening results of anti-HCV EIA. As a result, high index values of anti-HCV, high ALT and low albumin levels were related to the present HCV infection (57).

Eiji Tanka et al; (2000)

The HCV core antigen assay evaluated in this study was designed to provide clinical sensitivity comparable to genomic amplification assays and to quantities the level of antigen as an indirect measure of viral load. In addition, the number of steps required for pretreatment of samples was reduced from 6 to 1 as compared with the first generation core antigen assay (58).

Stephane Chevaliez, Jean-Michel Pawlotsky; (2006)

Conducted a study in which they use serological and virological tests has become essential in the management of hepatitis C virus (HCV) infection in order to diagnose infection, guide treatment decisions and assess the virological response to antiviral therapy. Virological tools include serological assays for anti-HCV antibody detection and serological determination of

the HCV genotype, and molecular assays that detect and quantify HCV RNA and determine the HCV genotype. Anti-HCV antibody testing and HCV RNA testing are used to diagnose acute and chronic hepatitis C. HCV RNA monitoring during therapy is used to tailor treatment duration in HCV genotype 1 infection, and molecular assays are used to assess the end-of-treatment and, most importantly the sustained virological response, i.e. the endpoint of therapy (59).

Batool A. Al-Haidary et al; (2007)

Conducted study which shows highly specificity & sensitivity of ELISA technique for HCV detection which results in 100% positivity by RIBA methods. Moreover, Viral load estimation shows that (71.9%) of HCV sera samples with viral load >615 IU /ml and only (28.1%) with viral load <615 IU/ml with highly significant difference between them (P=0.013). Furthermore, there is a highly significant variations between liver function test in comparison with control group (P< 0.01), while SGPT is the only parameter which significantly affected by viral load (P= 0.01 1). Besides that, neither INF-_ nor IL-12p40 level has been affected by viral loads more or less 6151U /ml. It was concluded that ELISA technique is still the best accurate reliable method for viral detection and SGPT is a good marker for highly viral loaded samples (60).

Muhammad Faisal Bashir et al; (2012)

Conducted study which shows that the male Pakistanis were more infected with HCV, as they were more exposed to risk factors such as blood transfusion, accidents, surgical operations, and shaving of beards with unsterilized instruments at barber salons. It was observed that ALT and AST was relatively more raised in HCV and diabetic group than HCV alone group. Similarly, significantly elevated levels of ALT and AST in diabetic HCV patients than non-diabetic HCV patients were reported elsewhere. Serum ALT was reported raised in 73.7% diabetic HCV patients than 18.5% diabetic patients without HCV infection. Abnormal liver function tests have been reported by HCV diabetic patients (61).

Rajesh K Sharma et al; (2016)

In India, serological identification of HCV infection is generally based on the detection of HCV-Antibody by ELISA / CLIA / Rapid tests and confirmed by supplemental assays like Recombinant Immuno-Blotting Assay (RIBA) or Line Immuno Assay (Innolia) and / or Nucleic Acid Test (NAT), which uses Polymerase Chain Reaction (PCR) technology for HCV-RNA detection. NAT detects HCV-RNA at very early stage followed by HCV core Ag or fourth generation HCV Antigen-Antibody test then HCV-Antibody test. The use of highly sensitive HCV-Antibody or HCV-Antigen assay or HCV Antigen-Antibody assay or HCV-RNA by NAT reduce the seroconversion window period and increase the opportunity of clinician to start treatment early after exposure of the HCV and prevent the public from HCV infection (63).

CHAPTER 3

MATERIAL AND METHODS

This prospective study was a hospital based study conducted in Mannat Hospital, Northern India (Jalandhar, Punjab) over a period of four months. This study included 60 cases. Over all the cases were in the age group of 20-70. Both genders are included for this i.e. male and female. The number of males were 39 and number of females were 21.

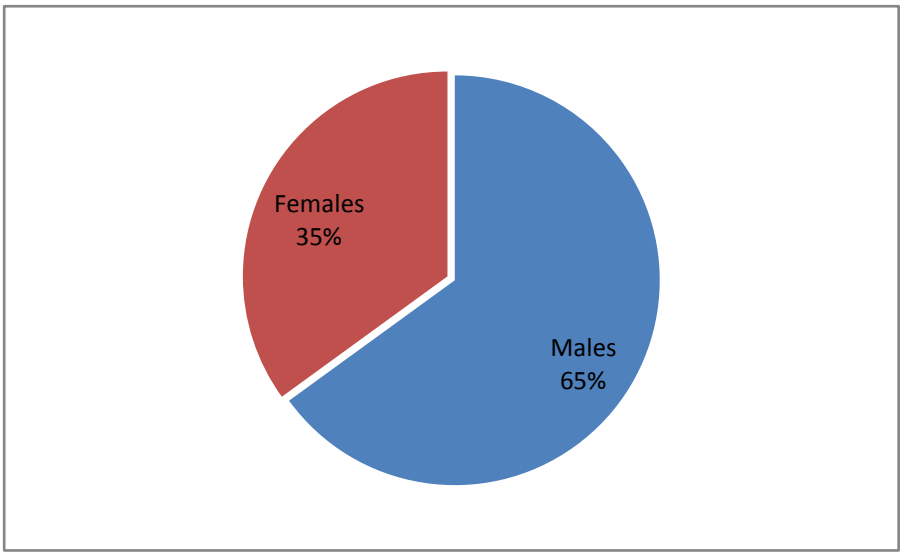

Figure 6: Pie chart showing number of male and female patients

3.1 SAMPLES

1. Blood samples

Sterile syringe was used to draw blood. Blood samples were collected from all patients by vein puncture the blood was treated immediately with EDTA-K2 for platelets count. Sera were separated from the rest of blood samples and tested fresh for liver function test.

Routine blood pictures including platelet counting were determined by XP-100 Sysmes automated hematology analyzer (Sysmes Corporation, Japan).

2. Liver biopsy and blood collection

Needle liver biopsy specimens (n =) were taken from the patients and examined by a pathologist unaware of the laboratory results. Biopsies were processed for diagnostic purposes. Fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4 µm thick, routinely stained with hematoxyline and eosin.

3 .Biochemical determination of liver function tests:

Serum were processed for the biochemical analysis of albumin, bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) according to the manufacture instructions. All serum and tissue samples were obtained with informed consent.

3.2 PROCESSING OF THE SAMPLE

Samples were received with a prescription form of the patient and the entry of the sample was done in separate register as their type and unique ID was given to the sample. The unique Id was bearing all the information sent with the sample.

3.3 REQUIREMENT

- Syringes
- Test tube
- Tunicate
- Cotton swab
- Centrifuge
- Test tube rack
- Analyzer

3.4 METHOD FOR SGPT USING ERBA CHEM 5 CHEMISTRY ANALYSER SEMI AUTOMATED

SGPT (ALT, GPT) FS IFCC method without pyxidoxal-5-phosphate

Principle: This ALT/GPT regent is based on the recommendations of the IFCC without pyridoxal phosphate. This series of reaction involved in the assay system is as follows:

L-Alanine + 2-oxoglutarate $\xrightarrow{ALT}$ L-Glutamate + Pyruvate

Pyruvate + NADH +H+ $\xrightarrow{LDH}$ D Lactate + NAD+

1) The amino group is enzymatically transfered by SGPT / ALAT present in the sample from alanine to the carbon atom of 2-oxoglutarate yielding pyruvate and L-glutamate.

2) Pyruvate is reduced to lactate by LDH present in the reagent with the simultaneous oxidation of NADH to NAD. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due the oxidation of NADH.
3) Endogenous sample pyruvate is rapidly and completely reduced by LDH during initial incubation period to avoid interference during the assay.

Assay procedure:

Wave length: 340 nm, Hg 334 nm, Hg 365 nm

Optical path: 1 cm

Temperature: 37 C

Procedure:

1. Take a 500 ul SGPT reagent in ria vial.
2. Then add 50 ul serum sample in SGPT reagent.
3. Mix the sample, then aspirates the sample from sample probe.
4. The optical density of the reacting solution is taken at 340nm.
5. The SGPT activity is then calculated.

Calculation:

With calibrator:

$$\text{ALT (U/L)} = \frac{\text{A/min sample}}{} \quad \text{conc. Calibrator (U/L)}$$

Conversion Factor

ALT (U/L) 0.0167 = ALT (ukat/L)

Normal Value

ALT 0-40 U/

3.5 SGOT (ASAT, GOT) FS IFCC METHOD WITHOUT PYXIDOXAL-5-PHOSPHATE

Principle: This reagent is based on IFCC recommendations, without pyridoxal phosphate. The series of reactions involved in the assay system is as follows:

L- Aspartate + 2- Oxoglutarate ASAT L- Glutamate + Oxalacetate.

+Oxalacetate + NADH + H+ MDH L- Malate + NAD+

1. SGOT / ASAT present in the sample catalyses the transfer of the amino group from L-aspartate to 2-oxoglutarate forming oxaloacetate and L-glutamate.
2. Oxaloacetate in the presence of NADH and Malate dehydrogenase (MDH) is reduced to L-malate. In this reaction NADH is oxidized to NAD. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH to NAD.
3. Addition of Lactate dehydrogenase (LDH) to the reagent is necessary to achieve rapid and complete reduction of endogenous pyruvate so that it does not interfere with the assay.

Assay procedure:

Wave: 340 nm, Hg 334nm, Hg 365 nm

Optical path: 1 cm

Temperature: 37 C

Procedure:

1. Take a 500 ul SGOT reagent in ria vial.
2. Then add 50 ul serum sample in SGOT reagent.
3. Mix the sample, then aspirates the sample from sample probe.
4. The optical density of the reacting solution is taken at 340nm.
5. The SGOT activity is then calculated.

Calculation:

With calibrator:

ASAT (U/L) = A/min Muestra conc. Calibrator (U/L)

Conversion Factor

ASAT (U/L) 0.0167 = ASAT (ukat/L)

Normal Value

SGOT 0-40 U/L

3.6 ALKLAINE PHOSPHATE (FS), IFCC METHOD 37 C

Methodology: Kinetic photometric test, according to the international federation of clinical chemistry and laboratory medicine (IFCC).

Principle:

P- Nitrophenylphosphate + H 0 AP Phosphates + P- Nitrophenol.

At the pH of the reaction, 4-nitrophenol has an intense yellow colour. The reagent also contains a metal ion buffer system to ensure that optimal concentrations of Zinc and Magnesium are maintained. The metal ion buffer can also chelate other potentially inhibitory ions which may be present. The reaction is monitored by measuring the rate of increase in absorbance at 405 or 415 nm which is proportional to the activity of ALP in the serum.

Assay procedure:

Wave length: Hg 405nm, (400-420nm)

Optical path: 1 cm

Temperature: 37 C

Measurement: Against reagent blank.

Procedure:

1. Take a 500 ul ALP reagent in RIA vial.

2. Then add 10 ul serum sample in ALP reagent.
3. Mix the sample, then aspirates the sample from sample probe.
4. The optical density of the reacting solution is taken at 415nm.
5. The ALP activity is then calculated.

Calculation:

With calibrator

AP (U/L) = A/ min Sample conc. Calibrator (U/L

Calculation Factor

ALP (U/L) 0.0167 = ALP (ukat/L)

Normal Values

ALP 25-90 U

3.7 BILLIRUBIN TOTAL AND DIRECT

Principle: Principle Modified method of Pearlman & Lee in which a surfactant is used as a solubilizer. Bilirubin glucuronate reacts directly with sulphodiazonium salt and forms coloured derivative azobilirubin. The colour intensity of formed azobilirubin measured at 540 - 550 nm is proportional to direct bilirubin concentration in the sample.

Assay procedure:

Wave length: 546/630 (670) nm

Optical path: 1 cm

Temperature: 37 C

Procedure:

	Reagent blank	Standard(cal.)	Sample
Working reagent	0.500ml	0.500ml	0.500ml
Sample	-	-	0.025ml
Standard (cal.)	-	0.025ml	-
Distilled water	0.025ml	-	-

Mix and incubate 5 min. at 37 °C. Measure absorbance of the sample A sample and standard A standard against reagent blank.

Calculation:

With calibrator:

Total and Direct Billirubin (mg/dl) = ΔAsam/ ΔAst x Conc. Calibration

Normal Value

TOTAL BILIRUBIN:

Adults: 0 – 2.0 mg/dl

Cord < 2 mg/dl

Newborns,

Premature:

0 – 1 days 1.0 – 8.0 mg/dl

1 – 2 days 6.0 – 12.0 mg/dl

3 – 5 days 10.0 – 14.0 mg/dl

Newborns, full term:

0 – 1 days 2.0 – 6.0 mg/dl

1 – 2 days 6.0 – 10.0 mg/dl

3 – 5 days 4.0 – 8.0 mg/dl

DIRECT BILIRUBIN: Adults and infants: 0 – 0.2 mg/dl

3.8 TOTAL PROTEIN BY BIURET METHOD

PRINCIPLE: The peptide bonds of protein react with copper II ions in alkaline solution to form a blue-violet ion complex, (the so called biuret reaction), each copper ion complexing with 5 or 6 peptide bonds. Tartrate is added as a stabiliser whilst iodide is used to prevent auto-reduction of the alkaline copper complex. The colour formed is proportional to the protein concentration and is measured at 546 nm (520-560).

Assay procedure:

Wave length: 520-560 nm

Optical path: 1 cm

Temperature: 37 C

Procedure:

	Reagent blank	Sample	Standard(calibrator)
Reagent R1	1.00ml	1.00ml	1.00ml
Distilled water	0.02ml	-	-
Sample	-	0.02ml	-
Standard(calibrator**)**	-	-	0.02ml

Mix and incubate for 10 minutes (in case of automatic procedure incubate for 5 minutes) incubation in the dark. Absorbance of the sample A1 and the standard (calibrator) A2 against reagent blank is read in interval 30 minutes.

CALULATION:

Total protein (g/dl) = A1/A2 x Cst Cst = standard (calibrator) concentration

NORMAL VALUE

TOTAL PROTEIN 6.4 – 8.3 g/d

3.9 ALBUMIN BY BROMO CRESOL GREEN METHOD

Principle: Albumin binds with Bromo Cresol Green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue-green colour formed is proportional to the concentration of albumin, when measured photometrically between 540–630 nm with maximum absorbance at 625 nm.

Assay procedure:

Wave length: 578(540-630) nm

Optical path: 1 cm

Temperature: 37 C

Procedure:

	Reagent blank	Standard(cal.)	Sample
Reagent 1	1.00ml	1.00ml	1.00ml
Sample			0.01ml
Standard(cal.)		0.01ml	
Distilled water	0.01ml		

Mix and incubate 1–5 min. at 37 °C.Measure absorbance of the sample A and standard Ast against reagent blank.

Calculation:

Albumin (g/dl) = ΔAsam/ ΔAst x CST Cst = standard (calibrator) concentration

Normal value

Albumin 3.5 - 5.2 g/ml

HCV RNA Quantitative and HCV Genotyping send to reference laboratory. This test is done by Real time PCR method.

3.10 STATICAL ANALYSIS

All the statical analysis was performed using SPSS software and Microsoft excel 2007

CHAPTER 4

RESULT AND DISCUSSION

During the study period in the Mannat Hospital in Jalandhar, Clinical samples were obtained from patients infected with HCV. A total number of patients are 60. Among them positive and negative HCV samples were listed below:

Table: Distribution of HCV Positive and Negative

Table 1: Percentage and number of HCV positive and negative subjects

Samples	No of samples	Percentage
Hepatitis C Positive	39	65%
Hepatitis C Negative	21	35%

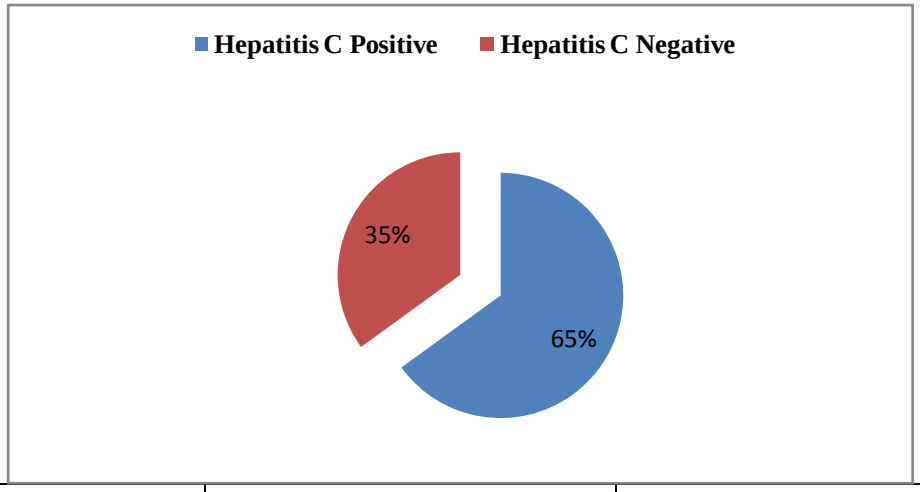

Figure 7: Pie chart showing % of HCV positive and negative patients

Table 2: HCV positive Male and Female

SEX	HCV POSITIVE
Male	29
Female	10

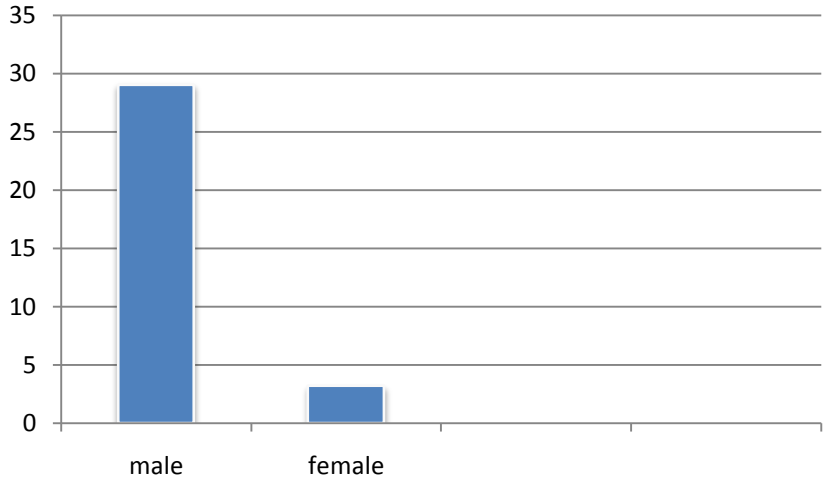

Figure 8: Showing Number of HCV positive males and females

4.1 Comparison of Serum SGOT among HCV patients and Control (Male)

Table 3: Mean level and S.D. of SGOT in HCV positive patients and control Subjects (Male)

	MEAN ± SD
HCV Positive Patients	69.21 ± 37.27 IU/L
Control	23.00 ± 6.33 IU/L

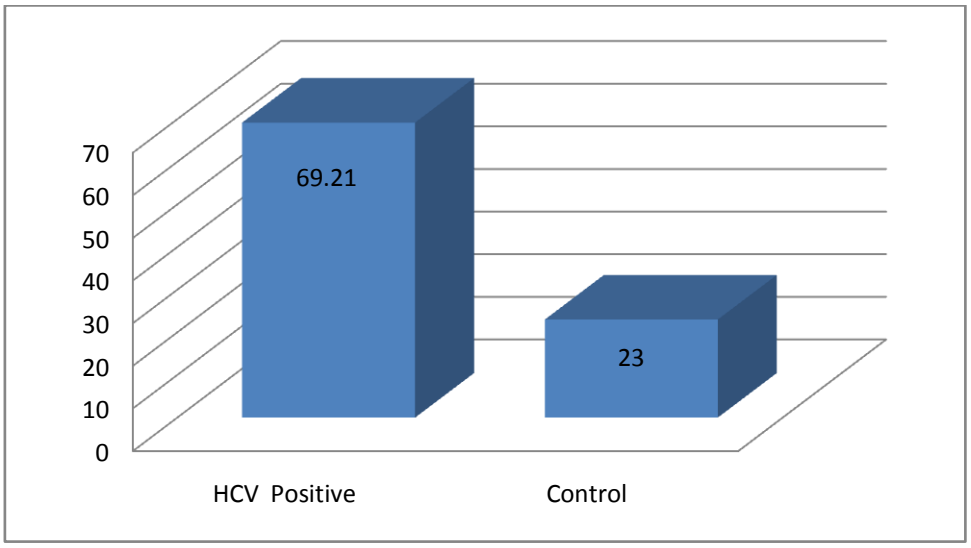

Figure 9: Mean level of SGOT in HCV positive patients and control Subjects (Male)

Mean of level of SGOT in HCV positive patients was 69.21 IU/L with standard deviation of ±37.27 IU/L. In control subjects mean level of SGOT was 23 IU/L with S.D. ±6.33 IU/L.

4.2 Comparison of Serum SGPT among HCV patients and Control (Male)

Table 4: Mean level and S.D. of SGPT in HCV positive patients and control Subjects (Male)

	MEAN ± SD
HCV Positive Patients	93.24 ± 55.98
Control	25.30 ± 6.75

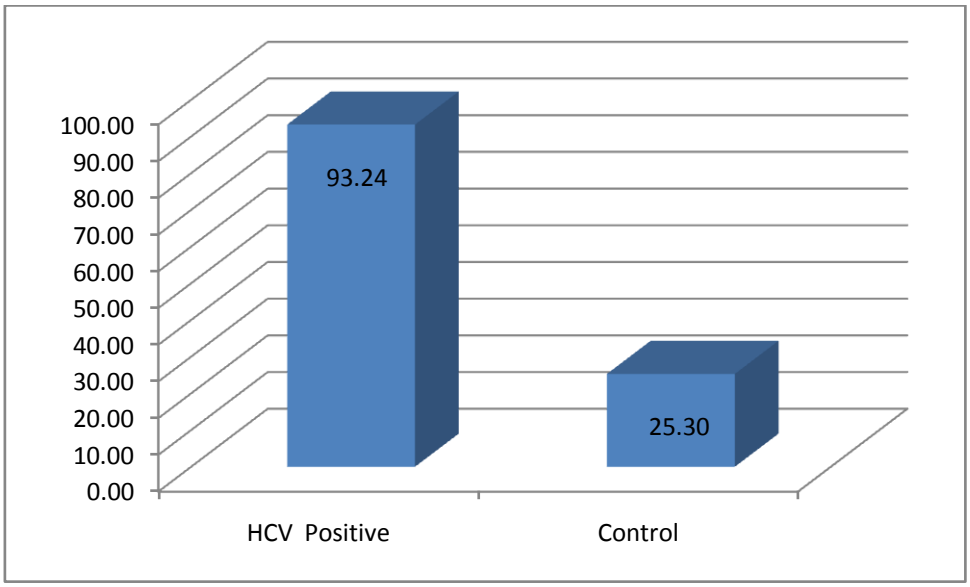

Figure 10: Mean level of SGPT in HCV positive patients and control Subjects (Male)

Level of SGPT was high in HCV positive patients than control subjects. Mean level of SGPT was 93.24 IU/L with ±55.98 standard deviation. In control subjects 25.30±6.75 is mean and S.D. level of SGPT.

4.3 Comparison of Serum ALP among HCV patients and Control (Male)

Table 5: Mean level and S.D. of ALP in HCV positive patients and control Subjects (Male)

	MEAN ± SD
HCV Positive Patients	73.03 ± 17.83
Control	53.00 ± 3.37

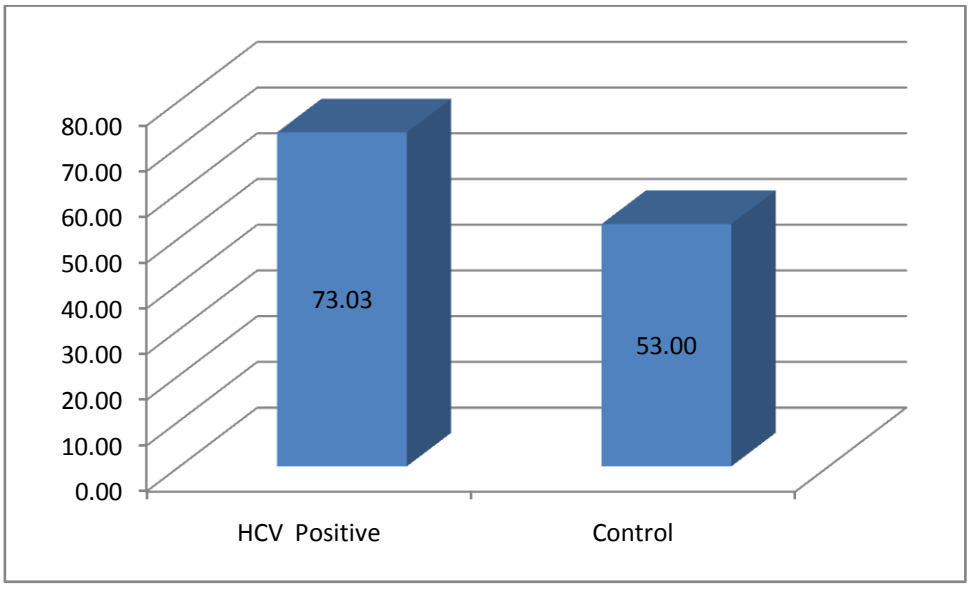

Figure 11: Mean level of ALP in HCV positive patients and control Subjects (Male)

ALP level 73.03 IU/L is the mean level of ALP in HCV positive subjects with S.D. of ±17.83 IU/L. in case of control subjects mean ALP level was 53.0 IU/L with ±3.37 IU/L S.D.

4.4 Comparison of Serum Protein among HCV patients and Control (Male)

Table 6: Mean level and S.D. of serum protein in HCV positive patients and control Subjects (Male)

	MEAN ± SD
Patients	7.25 ± 0.24
Control	7.38 ± 0.11

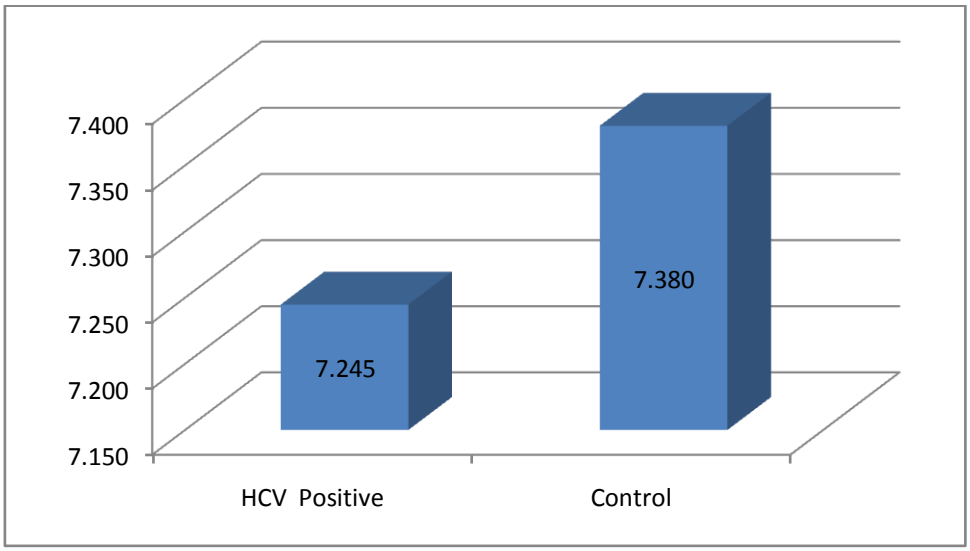

Figure 12: Mean level of Serum Protein in HCV positive patients and control Subjects (Male)

Mean level of total protein in HCV positive patients was 7.25 mg/dl with S.D. ±0.24 and in control male subjects 7.38 with ±0.11 S.D.

4.5 Comparison of Serum Albumin among HCV patients and Control (Male)

Table 7: Mean level and S.D. of serum albumin in HCV positive patients and control Subjects (Male)

	MEAN ± SD
Patients	3.33 ± 0.32
Control	3.64 ± 0.14

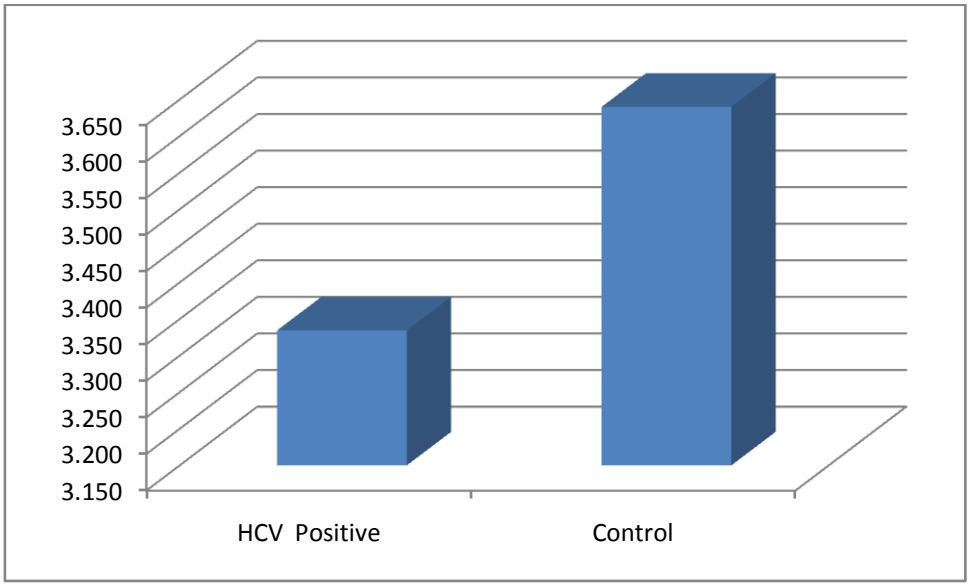

Figure 13 Mean level of Serum Albumin in HCV positive patients and control Subjects (Male)

Mean level of serum albumin in HCV positive patients was 3.33 mg/dl and S.D. was 0.32 mg/dl

4.6 Comparison of Serum SGOT among HCV patients and Control (Female)

Table 8: Mean level and S.D. of SGOT in HCV positive patients and control Subjects (Female)

	MEAN ± SD
Patients	55.00 ± 32.38
Control	20.00 ± 4.000

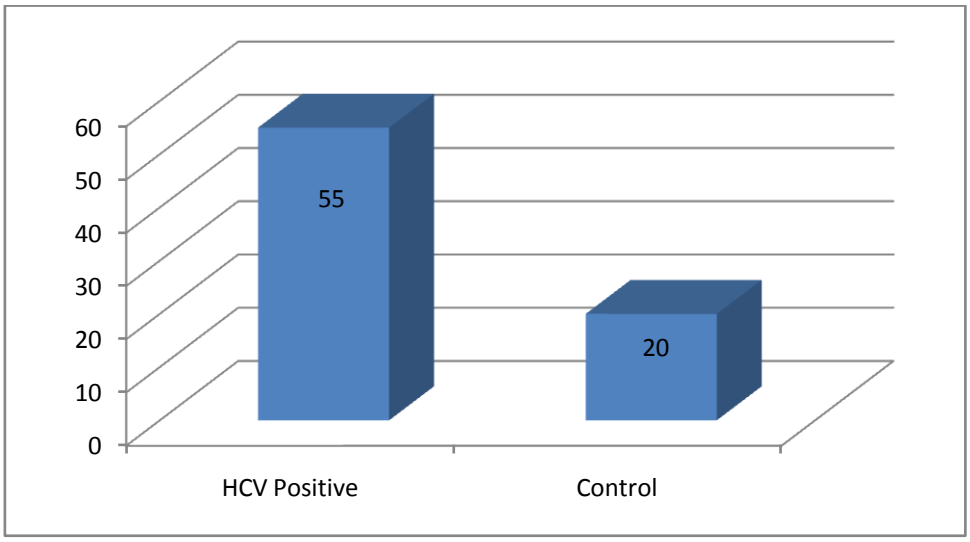

Figure 14: Mean level of Serum SGOT in HCV positive patients and control Subjects (Female)

Mean of level of SGOT in HCV positive patients was 55.00 IU/L with standard deviation of ±32.38 IU/L. In control subjects mean level of SGOT was 20 IU/L with S.D. ±4.0 IU/L. Level of SGOT was more in HCV positive patients whereas its level in within normal limit in control subjects.

4.7 Comparison of Serum SGPT among HCV patients and Control (Female)

Table 9: Mean level and S.D. of SGPT in HCV positive patients and control Subjects (Female)

	MEAN ± SD
Patients	65.40 ± 37.810
Control	21.73 ± 4.199

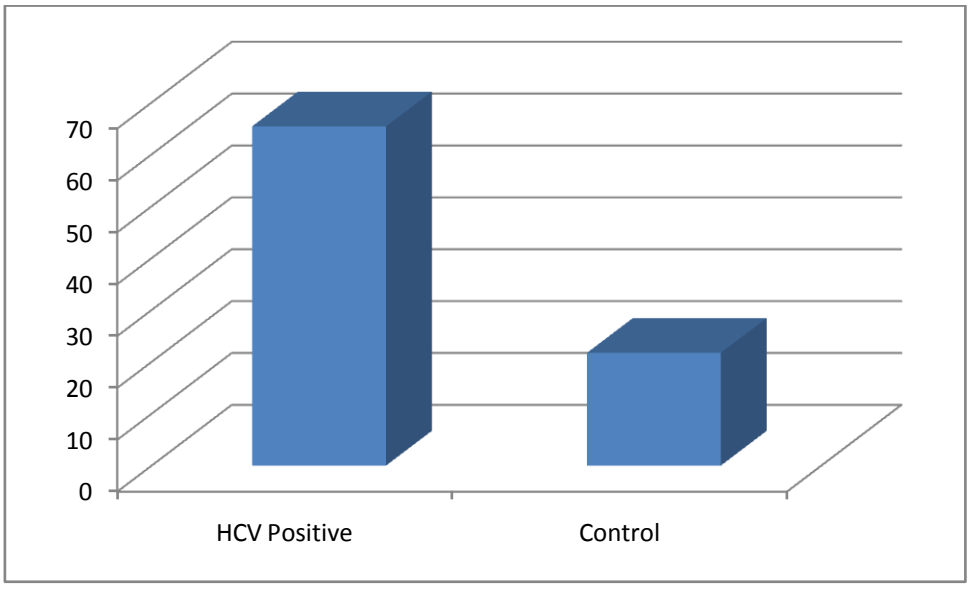

Figure 15: Mean level of Serum SGPT in HCV positive patients and control Subjects (Female)

This shows that there is statistically significant difference in serum SGPT between Hepatitis C positive patients and Control.

Level of SGPT was high in HCV positive patients than control subjects. Mean level of SGPT was 65.40 IU/L with ±37.81 standard deviation. In control subjects 21.73±4.19 is mean and S.D. level of SGPT.

4.8 Comparison of Serum ALP among HCV patients and Control (Female)

Table 10: Mean level and S.D. of ALP in HCV positive patients and control Subjects (Female)

	MEAN ± SD	P VALUE
Patients	78.80 ± 17.55	0.000
Control	21.73 ± 4.87	0.001

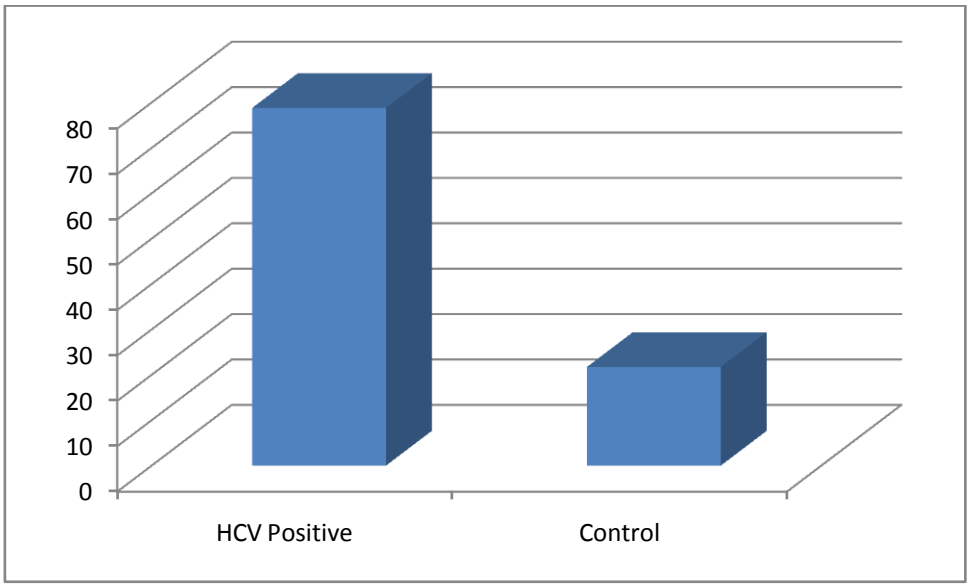

Figure 16 Mean level of Serum ALP in HCV positive patients and control Subjects (Female)

This shows that there is statistically significant difference in serum ALP between Hepatitis C positive patients and Control.

If we talk about ALP level 78.80 IU/L is the mean level of ALP in HCV positive subjects with S.D. of ±17.55 IU/L. in case of control subjects mean ALP level was 21.73 IU/L with ±4.87 IU/L S.D.

4.9 Comparison of Serum Protein among HCV patients and Control (Female)

Table 11: Mean level and S.D. of Serum Protein in HCV positive patients and control Subjects (Female)

	MEAN ± SD	P VALUE
Patients	7.09 ± 0.2132	0.140
Control	7.23 ± 0.1954	0.142

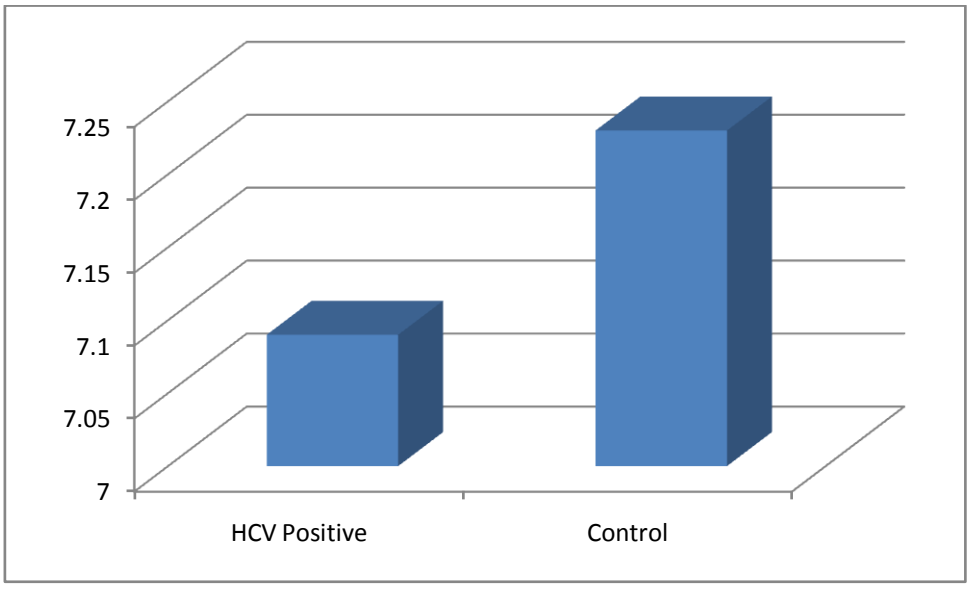

Figure 17: Mean level of Serum protein in HCV positive patients and control Subjects (Female)

This shows that there is no statistically significant difference in serum Protein between Hepatitis C positive patients and Control.

Mean level of total protein in HCV positive patients was 7.09 mg/dl with S.D. ±0.21 and in control male subjects 7.23 with ±0.19 S.D.

4.10 Comparison of Serum Albumin among HCV patients and Control (Female)

Table 12: Mean level and S.D. of Serum Albumin in HCV positive patients and control Subjects (Female)

	MEAN ± SD	P VALUE
Patients	3.26 ± 0.33	0.025
Control	3.52 ± 0.15	0.036

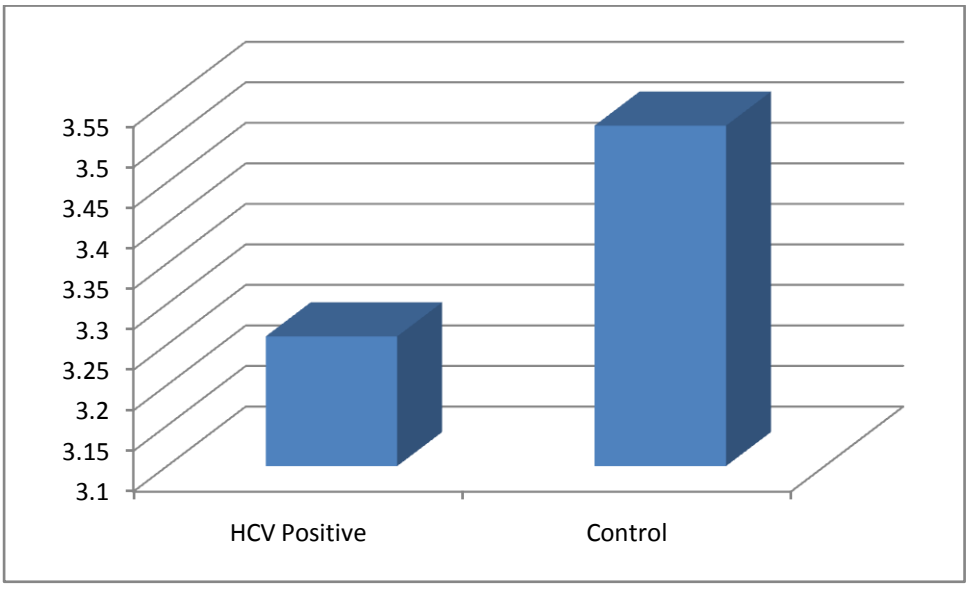

Figure 18: Mean level of Serum Albumin in HCV positive patients and control Subjects (Female)

This shows that there is statistically significant difference in serum Albumin between Hepatitis C positive patients and Control.

Mean level of serum albumin in HCV positive patients was 3.26 mg/dl and S.D. was 0.33 mg/dl and mean albumin level in control patients was 3.52 mg/dl with S.D. of 0.15 mg/dl.

CHAPTER 5

CONCLUSION

Liver is a vital organ in our body which performs various important functions in our body. HCV virus when infected a person it mainly affect liver. Damage to hepatocytes estimated by estimating various biochemical parameters. So prove relation between biochemical parameters and liver damage a study was conducted on HCV positive and control subjects.

This study shows level of SGOT was more in HCV positive patients (both male and females) whereas its level in within normal limit in control subjects. Level of SGPT was very high in HCV positive patients than control. Level of SGOT and SGPT in HCV positive and control subjects showes significant difference. High level of these parameters indicates damage to liver cells. Level of ALP is high in HCV positive patients as compare to normal subjects but still ALP level is within its normal limit. ALP level usually rises in biliary track obstruction, so HCV doesn't cause ant king of obstruction in bile duct. If we compare level of total protein and albumin, level of total protein is within normal limit but level of albumin slightly low than normal limit. This indicates HCV also effect protein synthesis in body due to liver damage.

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