UNIVERSITY OF IOANNINA
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Collagen morphometric analysis in liver biopsies of patients with chronic viral hepatitis (HCV or HBV) predicts virological response

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Ζ.Τ.

PREFACE

Machine learning focuses on how computers learn from the existing data with the use of statistics and computing algorithms. One of the main advantages of machine learning is that it constitutes an unbiased method of assessing information.

Predicting outcomes and recognizing features is already widely used in fields such as astronomy, finance and biology. Over the past two decades the use of computer-assisted analysis of biomedical data has dramatically increased. Machine learning techniques are constantly improving.

In medicine there are still some burdens, especially in trusting machines to complete a task that humans can do at high accuracy, as that of a human expert. Therefor, perhaps man-and-machine tactic might be of benefit.

CONTENTS

List of abbreviations

ALP: Alkaline Phosphatase
ALT: Alanine Transferase
anti-HBc: total Hepatitis B core Antiboby
APRI: AST-to-Platelet Ratio Index
ARFI: Acoustic Radiation Force Impulse
AST: Aspartate Transaminase
CCC: Concordance Correlation Coefficient
CDS: Cirrhosis Discriminant Score
CPA: Collagen Proportionate Area
DAAs: Direct-acting Antiviral Agents
DIA: Digital Image Analysis
DM: Diabetes Mellitus
DNA: Deoxyribonucleic Acid
DT: Decision Trees
EIA: Enzyme Immunoassays
ETR: End of Treatment Response
EVR: Early Virological Response
FCM: Fuzzy C-Means
H&E: Hematoxilin and Eosin
HAI: Histology Activity Index
HBeAg: Hepatitis B e-Antigen
HBsAg: Hepatitis B surface Antigen
HBV: Hepatitis B Virus
HCC: Hepatocellular Carcinoma
HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus
HLA: Human Leukocyte Antigen
IA: Image Analysis
IFN: Interferon
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IVC: Inferior Vena Cava
KM: K-Mean
KNN: K Nearest Neighbor
MRE: Magnetic Resonance Elastography
NB: Naïve Bayes
NN: Neural Network
PCR: Polymerase Chain Reaction
PDGF: Platelet-Derived Growth Factor
RF: Random Forests
RGB: Red/ Green/ Blue
RVR: Rapid Virological Response
SVM: Support Vector Machines
SVR: Sustained Virological Response
TGF: Transforming Growth Factor
TSH: Thyroid Stimulating Hormone

A. INTRODUCTION AND AIM OF THE STUDY

CHAPTER A1: Liver Anatomy

In human body, liver is considered as the largest gland and the second largest organ following the skin. It has an impressive range of synthetic, metabolic, haemopoietic and immunological activities and capacity for regeneration. It has an average weight of 1,5Kg. The normal liver is wedge-shaped, has a burgundy color, smooth surface and is soft-to-firm to touch. It is located at the right hypodiaphragmatic area, occupies the epigastric area and extends into part of the left hypodiaphragmatic area. **(Figure 1)** It is mostly protected by the rib cage, covered by parietal peritoneum and coated by a connective tissue layer, the Glisson's capsule[1]. **(Figure 2)**

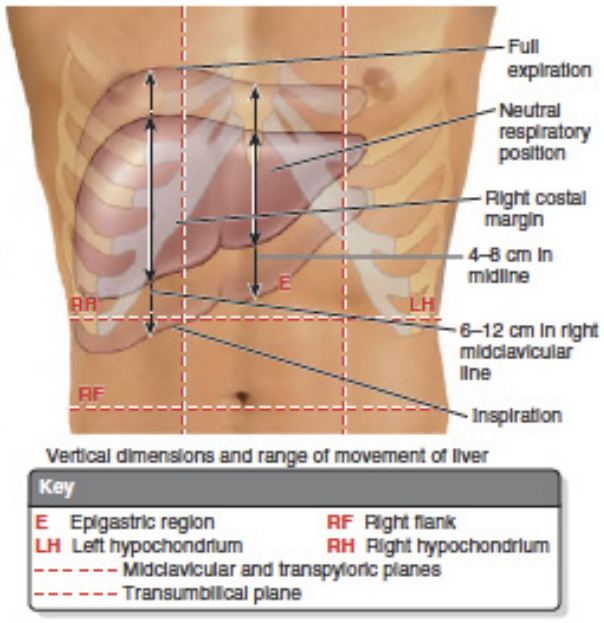

Figure 1: Surface anatomy of the liver. The liver's location, extent, relationship to the thoracic cage and range of movements with change of position and diaphragmatic excursion[2].

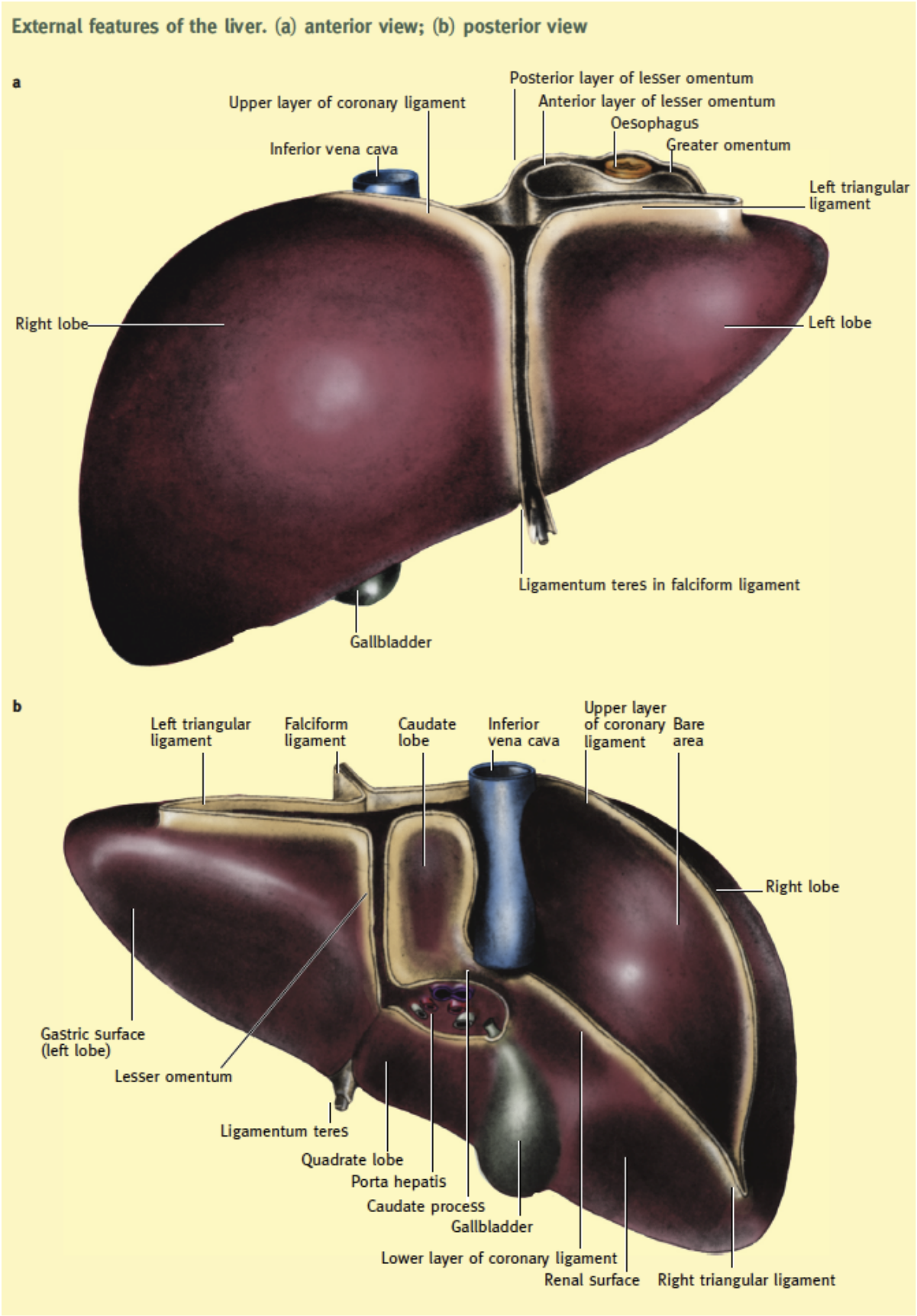

Figure 2: Anatomy of the liver[1].

The main gateway of the liver, or porta hepatis, is a transverse fissure through which the common hepatic artery and the hepatic portal vein are entering the liver and the common hepatic duct emerges from the liver. **(Figure 3)** Lymphatic vessels and a branch of the vagus nerve also pass through. The first three strictures are so called portal triad and each of them are subdivided to right and left branches.

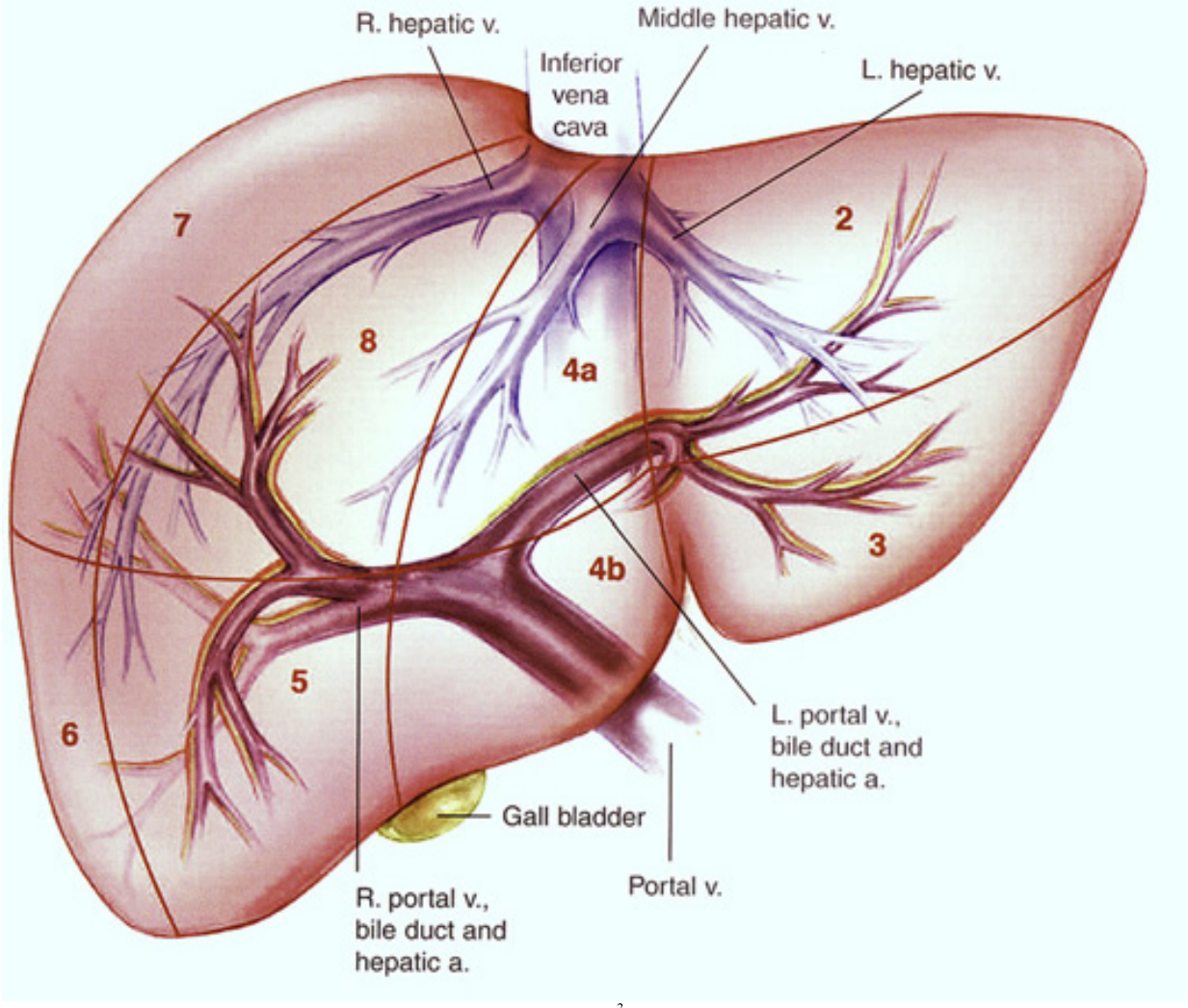

Figure 3: Intrahepatic vascular and biliary anatomy, anterior view[3].

The liver is traditionally anatomically divided into four lobes; the right, the left, the caudate and the quadrate[4]. **(Figure 4)** Surgeons prefer to divide it into eight segments based mostly on the vascular supply and biliary drainage[5]. **(Figure 5)** There are many other anatomical divisions, such as the hemilivers, divided in discrete 'halves' on a functional basis[1]. Proposed systems, though, are those of Couinaud, based on the distribution of portal and hepatic veins, and Healey and Schroy, based on the distribution of bile ducts[4].

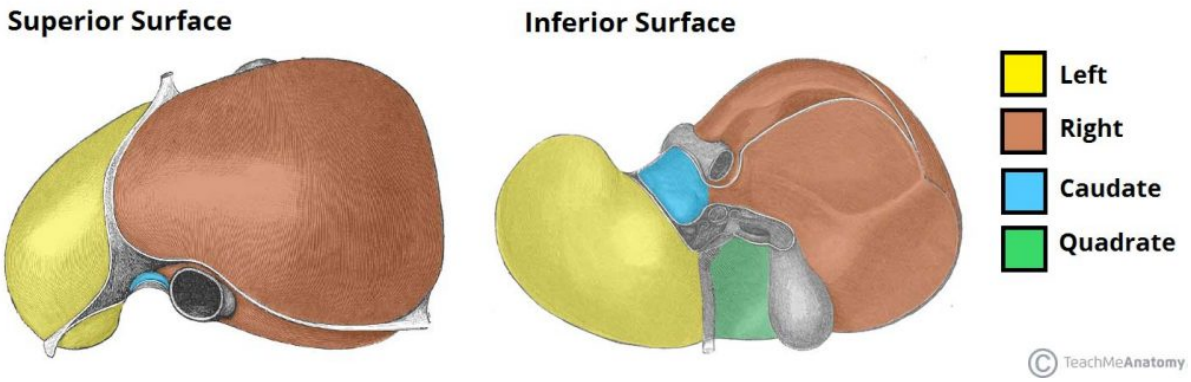

Figure 4: The anatomical lobes of the liver (google image - https://teachmeanatomy.info/abdomen/viscera/liver/)

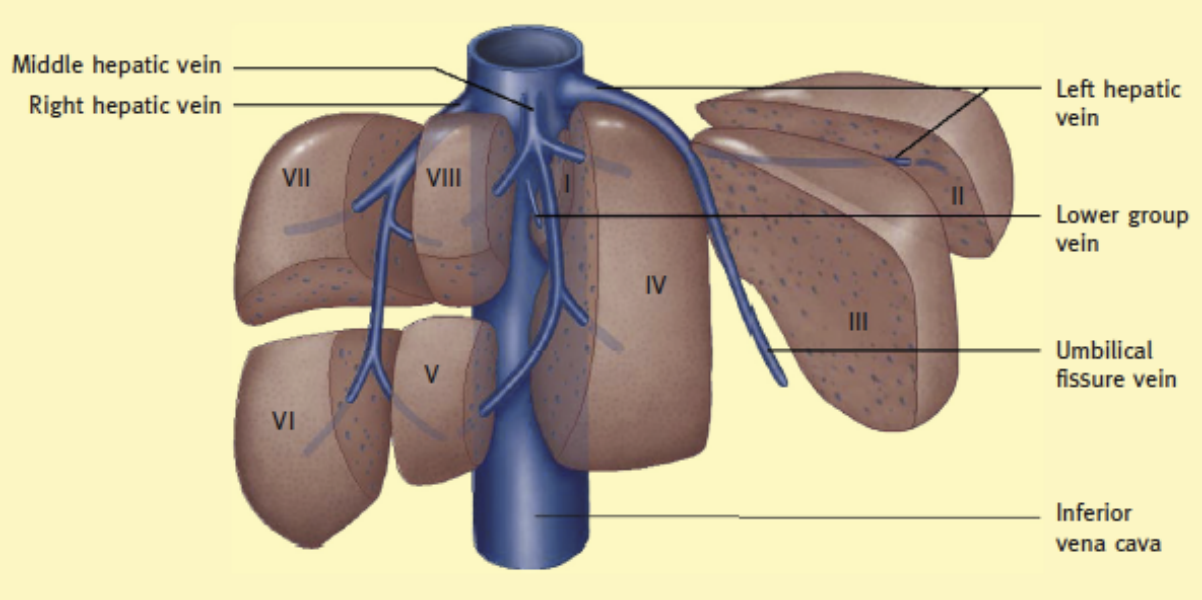

Figure 5: The location of the principal hepatic veins and the relationship of the hepatic veins to the functional anatomy of the liver[1].

The liver is subdivided into liver lobules, which are polygonal structures consisted of hepatocytes. Every one of the six corners of the lobules has a branch of portal triad[4].
Between the liver lobules there are the liver sinusoids. Within their walls there are stellate (or hepatic) macrophages the roll of which is to remove debris from the passing blood. In the center of the each lobule runs the central vein, which drains to the hepatic vein draining directly to the inferior vena cava (IVC). **(Figure 6)**

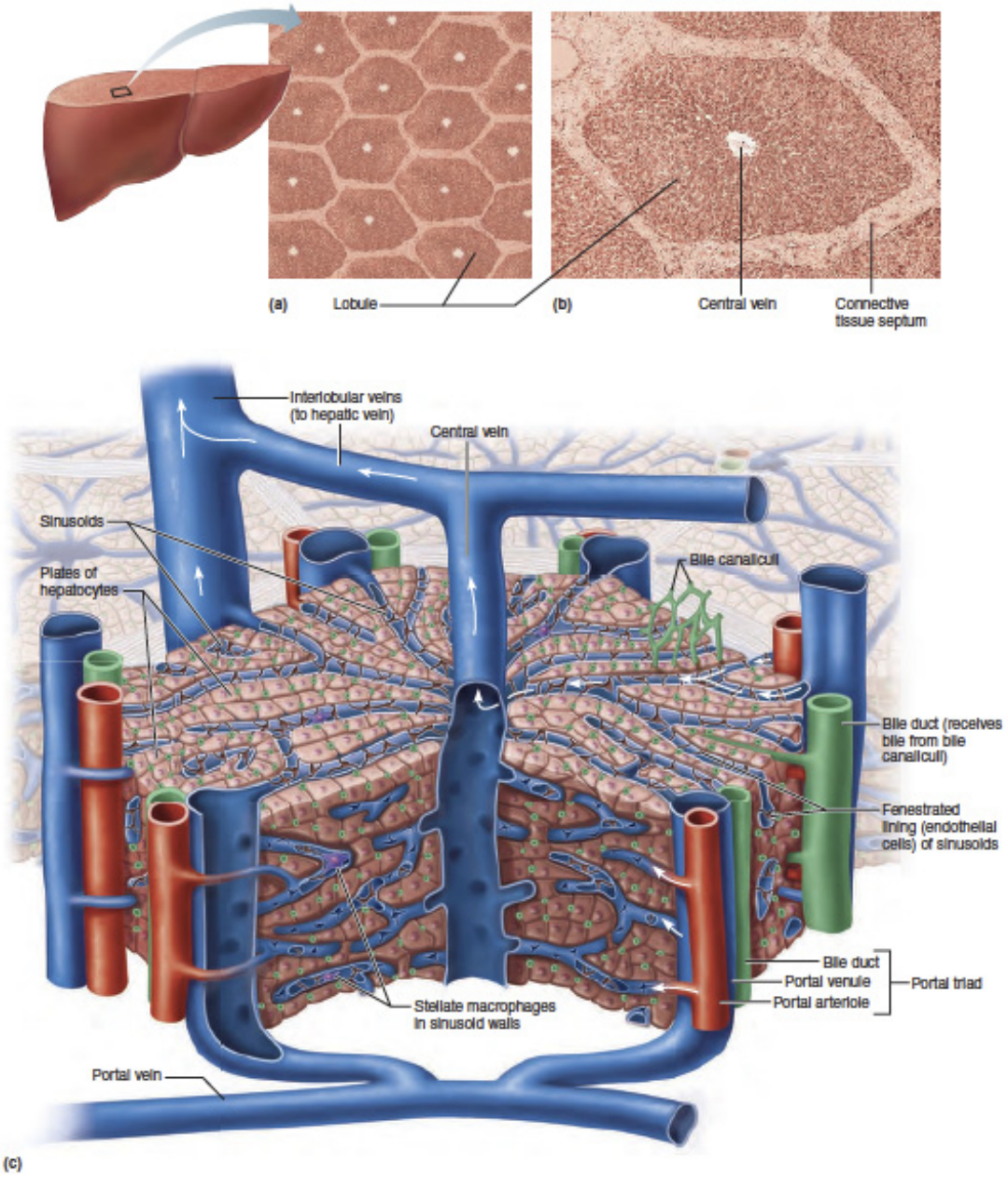

Figure 6: Microscopic anatomy of the liver. (a) Classic lobular pattern of a pig liver. (b) Enlarged view of one liver lobule. (c) Three-dimensional representation of a small portion of one liver lobule, showing the structure of sinusoids. Arrows indicate the direction of blood flow[5].

Blood and oxygen supply are mainly from the portal vein and the hepatic artery. About 1.5 liters of blood have been estimated being received by the liver per minute. The portal vein provides about 80% of the inflow with the remaining 20% being provided by the hepatic artery. This blood then enters the hepatic sinusoids, where it undergoes filtering including a considerable reduction in oxygen saturation, being an important source of oxygen to the hepatocytes. The deoxygenated blood returns to the circulation by the hepatic veins to the inferior vena cava[1].

Liver lymphatic channels are divided in superficial and deeply situated networks. The superficial lymphatic plexus lies inside the Glisson's capsule. The superficial lymphatics from the anterior part of the liver drain into the hepatic node, which transmits to the coeliac nodes, where most of the ones on the posterior part accompany the inferior vena cava, reach the posterior mediastinal lymph nodes and drain into the thoracic duct.
The deep liver lymphatics are also further subdivided to ascending and descending trunk. The ascending trunks drain to the posterior mediastinal lymph nodes and the descending trunks to the hepatic nodes[1].

The liver produces bile, which runs through the biliary ducts (right and left) that join to the common hepatic duct, which, together with the cystic duct, creates the common bile duct[2].

Innervation of the liver is consistent by sympathetic, parasympathetic (cholinergic) and adrenergic nerve fibers. Sympathetic and adrenergic fibers form a plexus mainly around the blood vessels and in a lesser extent the bile ducts along the sinusoidal walls[4]. **(Figure 7)**

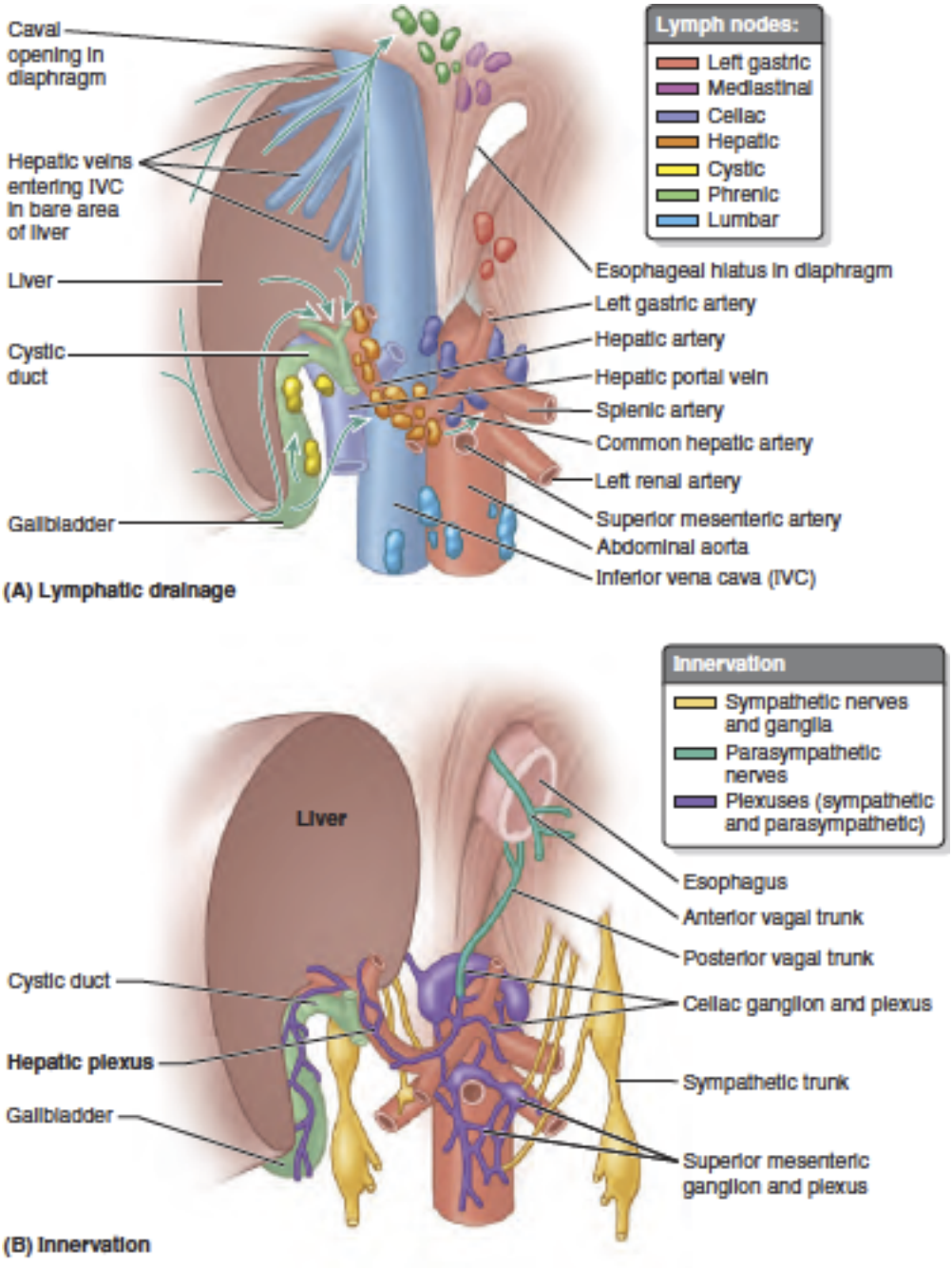

Figure 7: Lymphatic drainage and innervation of liver. A. The liver is a major lymph-producing organ. Lymph from the liver flows in two directions: that from the upper liver flows to lymph nodes located superiorly in the thorax; that from the lower liver flows to nodes located inferiorly in the abdomen. B. The hepatic plexus, the largest derivative of the celiac plexus, accompanies the branches of the hepatic artery to the liver conveying sympathetic and parasympathetic fibers[2].

CHAPTER A2: Liver Histology

Histologically the liver is divided into stroma and parenchyma. The stroma is consistent of connective tissue capsule, trabeculae and reticular network. The parenchyma is consistent of hepatocytes, blood vessels and bile ducts.

The hepatic lobule was classically described as a hexagon with a central vein at the center and portal tracts at three corners. **(Figure 6 and 8)** The main cells that the liver is compromised of are the hepatocytes. They constitute about 80% of the liver volume. Those are cells with a polygonal shape and either one have two spherical nuclei. They are arranged in plates and radiate from the center to the periphery. The hepatocytes cytoplasm is rich in mitochondria, lysosomes, well-developed Golgi apparatus and glycogen. These features indicate a high metabolic activity.

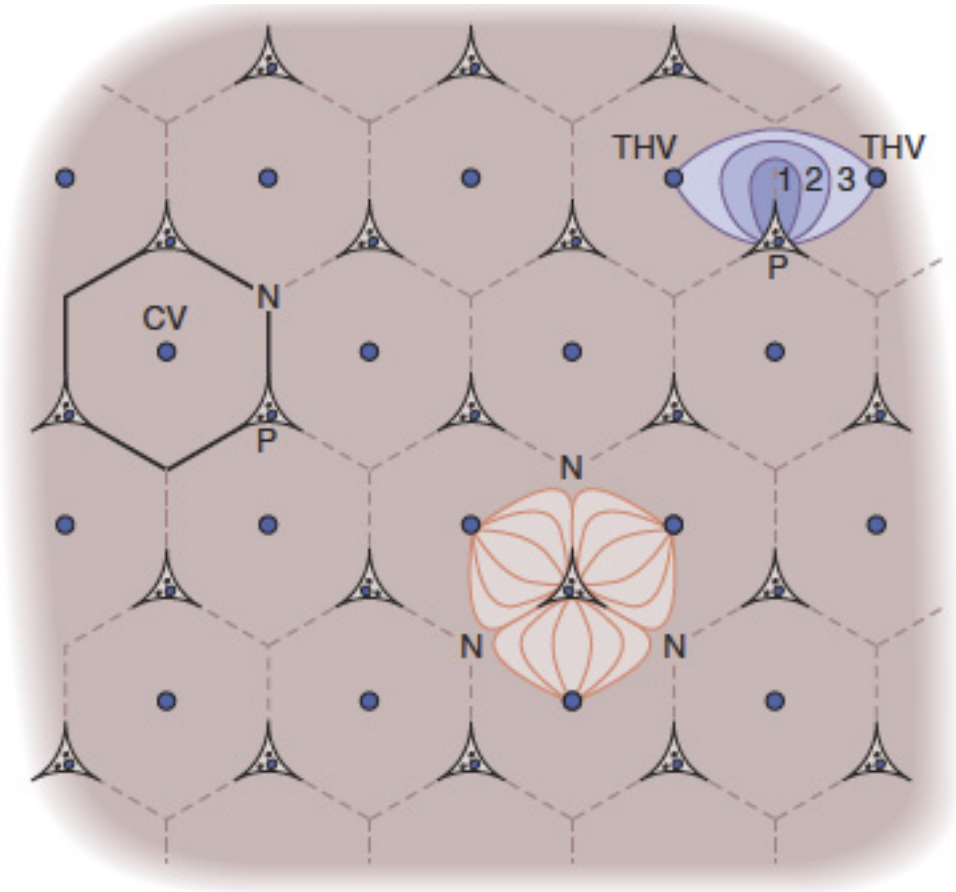

Figure 8: Schematic drawing of the liver architecture. At the left is the classic hepatic lobule, with the central vein as its center and portal tracts at three corners. Toward the middle is the portal unit, with the portal tract at its center, and central veins and nodal points at its periphery. At the right is the liver acinus, the center of which is the terminal afferent vessel and the periphery of which is drained by the terminal hepatic venule, or central vein. Zones 1, 2, and 3 extending from the portal tract to the terminal hepatic venule are shown. CV, central vein; N, nodal point; P, portal tract; THV, terminal hepatic venule[4].

Between the hepatic plates there are the sinusoidal cells, which are the prime barrier between hepatocytes and the blood. Those are mainly endothelial cells that have fenestrations, the size of which differs in the lobular periphery from that in the center. The pore size is controlled by contraction of actin filaments, which are within the endothelial cells.

One other type of cells that line sinusoids, are the Kupffer cells. Those are macrophage cells with a capacity to phagocytize large particles. Their activity, size and number are higher in the periportal region with a main role to detoxicate blood from senescent red blood cells and endogenous and exogenous substances.

Other perisinusoidal cells are the hepatic stellate cells. Those are also known as fat-storing cells, perisinusoidal lipocytes or "Ito cells". Their role includes vitamin A storage and fat metabolism. They also play significant role in hepatic fibrogenesis.

Between the sinusoids and the hepatocytes there is a space that contains plasma and collagen and scanty connective tissue and is called space of Disse, which acts as scaffold. **(Figure 9)** Between the periportal hepatocytes and portal connective tissue disease there is another space, the space of Mall. The first space passes the lymphatic fluid to the second, which then drains into the lymphatic vessels[4].

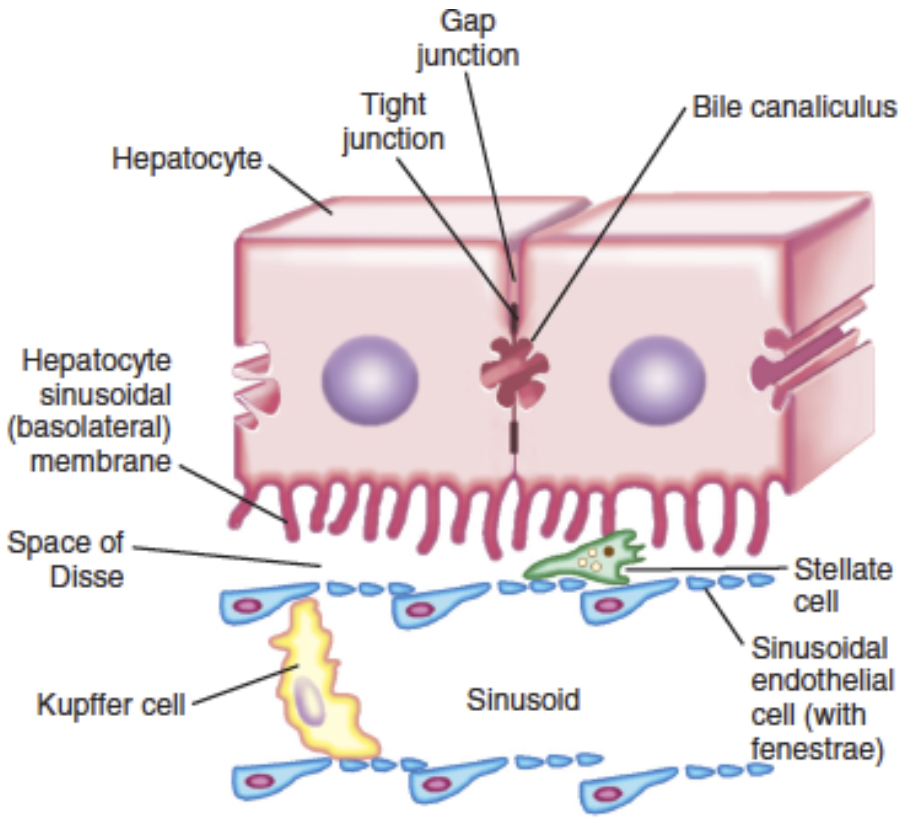

Figure 9: The spatial relationship among the different cell types of the liver. Sinusoidal plasma comes in direct contact with hepatocytes in the space of Disse. The endothelial cells are fenestrated and lack a basement membrane. Kupffer cells are located in the lumen of the sinusoid, where they are in direct contact with the sinusoidal endothelial cells and portal blood. Stellate cells are situated between the endothelial cells and hepatocytes, and come in direct contact with both cell types. The hepatocytes are joined with each other by tight junctions and the communicating gap junctions. The canalicular domain of the plasma membrane of two adjacent hepatocytes encloses the bile canaliculus[4].

The blood flow with the liver lobules is from the periphery to the center where bile flows in the opposite direction.

Another functional unit of liver is the hepatic acinus. This is defined by three concentric regions of hepatic parenchyma surrounding an artery in the center. **(Figure 10)** Functionally, based on oxygen supply, the liver acinus is divided into 3 zones. Zone 1 is around the portal tracts where the entrance of the oxygenated blood from hepatic arteries is. Zone 2 is the intermediate zone and zone 3 is adjacent to the central vein where oxygenation is poor.

One more functional liver unit is the portal lobule. **(Figure 10)** It has triangular shape and can be visualized by the imaginary space created by lines that connect central veins of three adjacent liver lobules. That contains a portal triad at the center. Each portal triad contains a bile duct branch, a hepatic artery branch, a portal vein branch and lymphatic channels embedded in connective tissue. There are also few lymphocytes, macrophages and mast cells.

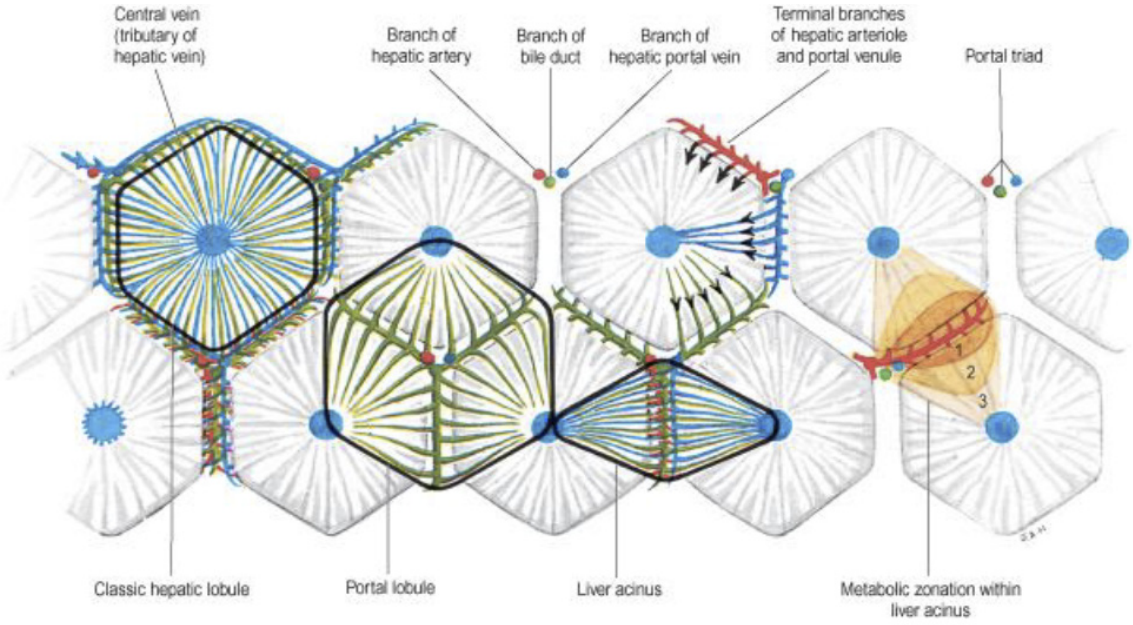

Figure 10: Portal lobule – functional liver unit[6].

The hepatocytes from the one side they border the sinusoid and from the other with canaliculus. That is an intercellular space with a diameter of about 1μm. Canals of Hering are the terminal canals were the bile canaliculi direct the bile to. Those are lined by hepatocytes and cholangiocytes and then pass the bile to the bile ductules, which are entirely lined by cholangiocytes. These are, in turn, connected further with the interlobular bile ducts, followed by the septal bile ducts connecting to the hepatic bile duct[4].

CHAPTER A3: Viral Hepatitis B

257 million people are estimated to be chronically infected worldwide by hepatitis B virus (HBV)[7]. This number has declined significantly since the introduction of universal vaccination in 1990s. Of those, 15% to 45% is expected to experience severe consequences such as liver cirrhosis or hepatocellular carcinoma (HCC) requiring liver transplantation or death.

Vaccines have been available since 1980s, though perinatal and early life exposures continue to be in the developing world major sources of infection. This is thought to be due to limited resources for vaccination on newborns[4].

HBV is more prevalent in sub-Saharian Africa and Southeast Asia. The mean incubation period is 60 to 90 days. The onset can be insidious or acute. It is most prevalent in young adults with sexual and percutaneous transmission as well as babies and toddlers[8]. The virus is 50 to 100 times more infectious than Human Immunodeficiency Virus (HIV) and 10 times as hepatitis C virus (HCV)[4].

Clinically HBV can occasionally be severe. Hepatitis fulminant can be seen in 0.1 to 1% of the infected individuals. 1 to 10% will progress to chronic hepatitis though in neonates that number reaches 90%. The prognosis has been found to be worse with age.

Pathogenesis and Natural History

The pathogenesis and severity of HBV-associated liver disease is considered to be related to the intensity of the host immunological response to the virus. This response encompasses both an innate and an adaptive immune response.

During the acute infection, the majority of the HBV DNA molecules are rapidly cleared from the liver by cytokines mediated mechanism of the innate immune system. In contrast, in chronic hepatitis B infection there is infrequent and weak HBV-specific T-cell response[4].

The natural history of HBV infection has been described to have four phases: the immune tolerance, the immune clearance, the inactive carrier and the reactivation phase. All four phases are more apparent in patients with acquisition of chronic hepatitis B early in life[4]. **(Figure 11)**

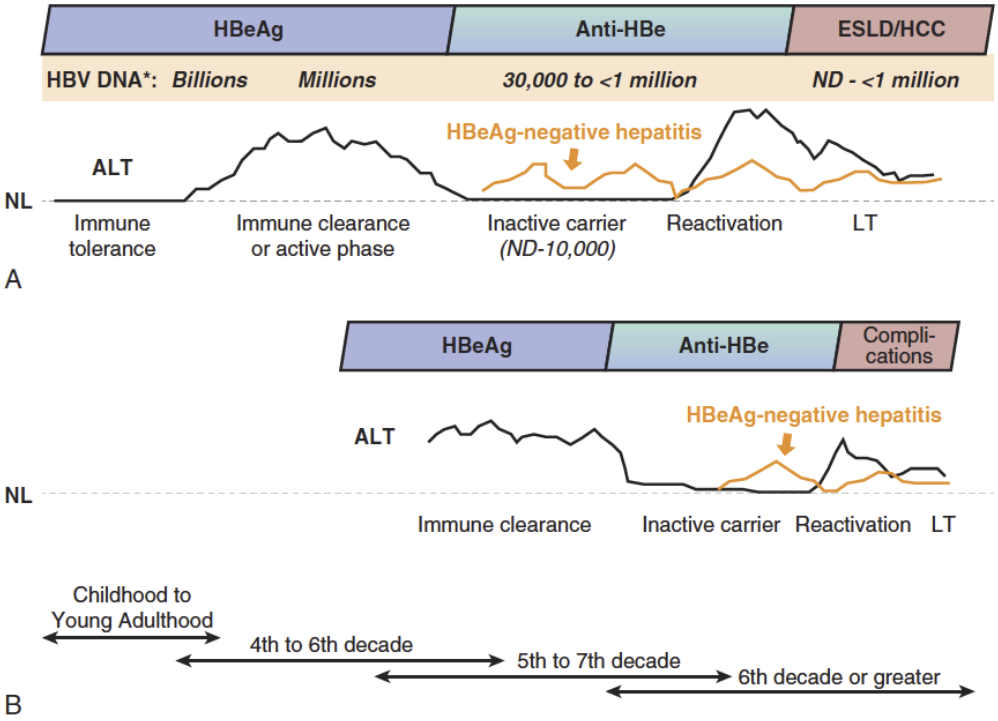

Figure 11: Natural history of chronic HBV infection[4].

If hepatitis B remains untreated on the active phase, cirrhosis might develop in up to 20% of the cases. Risk factors are older age, male gender, the stage of fibrosis at presentation as well as combined infection with other hepatitis or HIV as well as concomitant alcohol abuse. After the development of cirrhosis, hepatic compensation may develop in 5% to 8% of the cases and HCC in 2% to 4% of the cases. Population-based Asian cohort studies have shown that the serum HBV DNA level is the single best predictor of future progression to cirrhosis and HCC[4]. **(Table 1)**

PHASE	1	2	3	4
New terminology	HBeAg positive Chronic *infection*	HBeAg positive Chronic hepatitis	HBeAg negative Chronic *infection*	HBeAg negative Chronic hepatitis
Old terminology	*Immune tolerant*	*HBeAg-positive CHB*	*Inactive carrier*	*HBeAg-negative CHB*
HBsAg	High	High/Intermediate	Low	Intermediate
HBeAg	Positive	Positive	Negative	Negative
HBV DNA	>10E7 IU/mL	10E4-10E7 IU/mL	<2,000 IU/mL*	>2,000 IU/mL
ALT	Normal	Elevated	Normal	Elevated**
Liver disease	None/minimal	Moderate/severe	None	Moderate/severe
Disease progression	Low	**Moderate to high**	No, very low	**Moderate to high**
Treatment	Not indicated***	**Indicated**	Not indicated	**Indicated**

* HBV-DNA levels can be between 2,000 and 20,000 IU/mL in some patients without signs of chronic hepatitis
** Persistently or intermittently
*** Treatment is indicated in some patients

Table 1: Natural history of HBV and treatment indications[9].

Symptoms of acute infection include nausea, vomiting, malaise, as well as fever, arthralgia or arthritis and rash (most commonly maculopapular or urticarial) in about 10% to 20% of the patients. Jaundice is a feature in only 30% of the patients.

Usually signs and symptoms together with elevated serum ALT levels and HBsAg titers disappear in most of the cases after 1 to 3 months of the onset of illness[4]. **(Figure 12)**

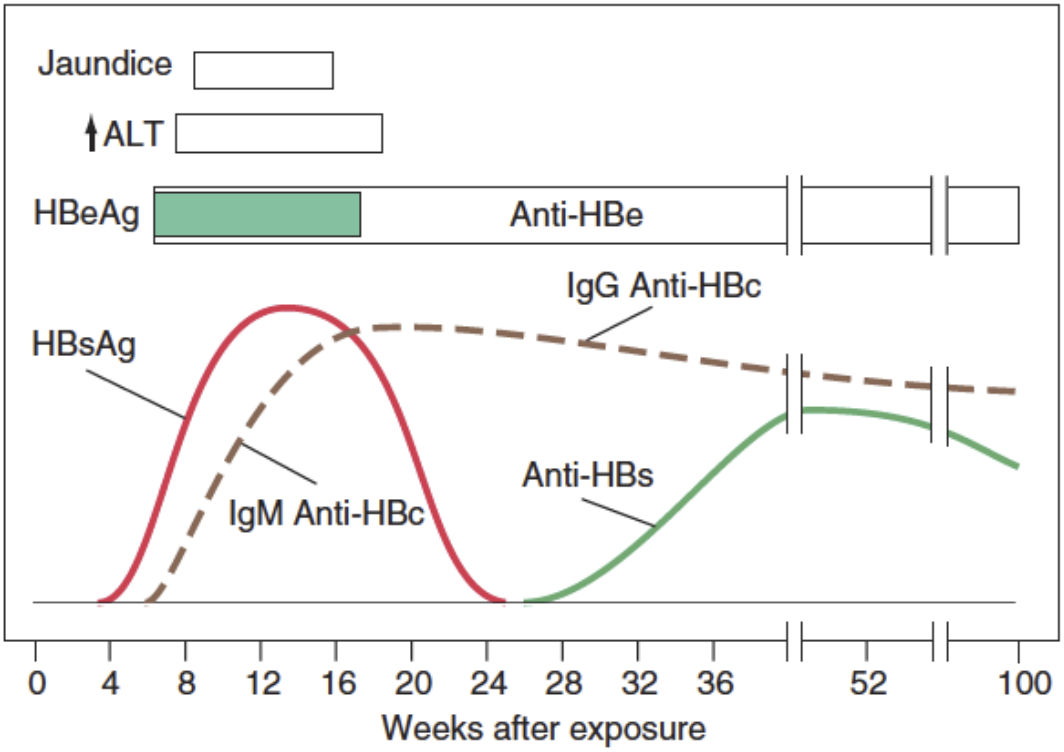

Figure 12: Scheme of typical clinical and laboratory features of HBV. ALT, alanine and aminotransferase[8].

During acute illness, levels of 1000 to 2000 U/L are typical for serum aminotransferases. ALT levels tend to be higher that AST levels. Regarding prognosis, that tends to be mostly correlated with the prothrombin time rather than the aminotransferase levels[4].

Patients who develop chronic HBV infection usually lack a history of acute hepatitis. They might remain symptom free even during reactivation periods. In physical examination, hepatosplenomegaly may be evident and in the development of decompensated cirrhosis common clinical signs are jaundice, spider telangiectasias, ascites and peripheral oedema. In prolongation of the prothrombin time, alongside with hypoalbuminemia in absence of nephropathy, and hypersplenism, someone should suspect progression to cirrhosis. In advanced cirrhosis serum AST levels are higher that ALT.

Acute flares play an important role on the natural history of the disease as, if they occur repeatedly, can lead to histological progression[4].

Diagnosis

HBsAg can be detected in serum 2 to 10 weeks after exposure to the virus and it is usually before the onset of the symptoms and elevation of ALT. If HBsAg remains detectable for over than 6 months, that implies evolution to chronicity.

Several weeks after the disappearance of HBsAg from the serum follows the appearance of anti-HBs, which can persist for life and provide long-term immunity[4].

During the window period that HBsAg has disappeared and anti-HBs has not appeared yet, the diagnosis is made by the detection of IgM anti-HBc, which usually remains for 4 to 6 months after an acute episode and can be used as a surrogate for active viral replication. **(Figure 12)** IgG anti-HBc is normally found during the recovery period from acute hepatitis, as well as during exacerbations of chronic infection. Anti-HBc can be isolated in serum of 1-4% of general population on low endemic areas and up to 50% in high endemic areas with only 10-30% having detectable HBV DNA. Understandably, recognizing false positive test results has very high clinical importance[4].

HBeAg is another viral protein that can be detected early in serum in acute HBV infections. It normally disappears around the time that serum aminotransferase levels peak, but if persistent that indicates high chance of chronicity. If found in serum of HBsAg-positive carrier, that indicates high level of viral replication and infectivity[4].

The DNA of the hepatitis B virus is mostly measured with quantitative PCR method and it is used to evaluate if a patient is a candidate for antiviral therapy, as well as monitor response to treatment. Qualitative PCR methods are also available and can be an even more sensitive method though, that has a limited clinical role and the technique lacks from standardization.

Histopathological features in hepatitis B

Periportal mononuclear cell infiltration is a characteristic feature in chronic HBV infection. Inflammatory cells can be seen between collagenous extensions from the portal tracts and liver parenchyma, referred as active septa. Lobular inflammation is more prominent during reactivated HBV.

Specific histologic feature of chronic hepatitis B seen on light microscopy is the presence of ground glass hepatocytes. That is a result of accumulation of HBsAg particles that are reach in cysteine which increases the affinity in certain dyes, such as Victoria blue, aldehyde

fuchsin and orcein. HBcAg were also found in the hepatocyte nuclei with electron microscopy and immunofluorescence studies[4]. **(Figure 13)** It is important to note that after successful treatment of HBV infection (with a nucleoside analog), the cytoplasmic core antigen often disappears, but nuclear core antigen staining may remain due to persistence of HBV cccDNA transcriptional template.

Steatosis is a feature of chronic hepatitis C rather than hepatitis B.

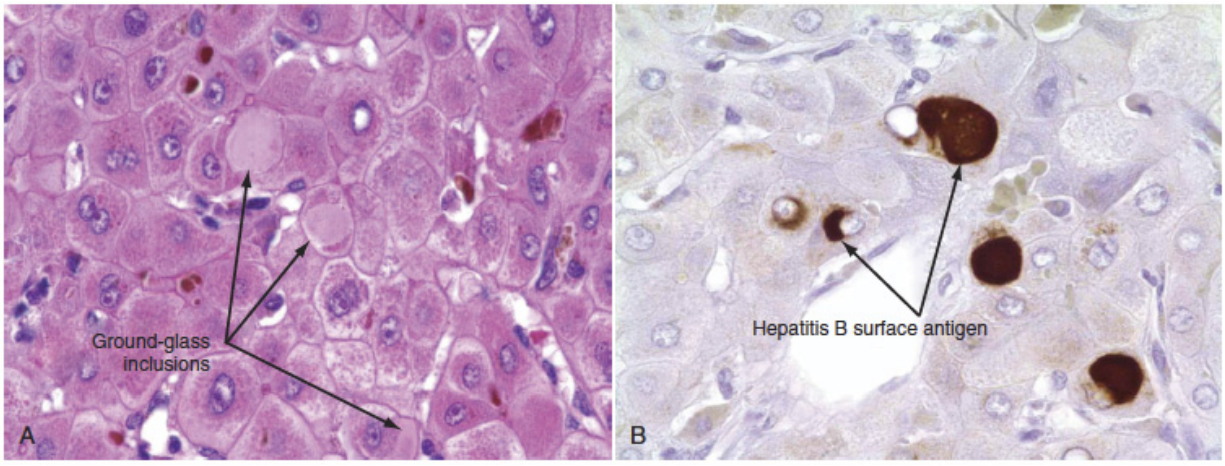

Figure 13: Histopathology of HBV infection. A: Ground-glass inclusions in hepatocytes. These represent large amounts of HBsAg (hepatitis B surface antigen) in the endoplasmic reticulum. (H&E x630). B: Immunohistochemistry for HBsAg[4].

Treatment

There are seven approved drugs available for the treatment of chronic hepatitis B, five of which are nucleoside analogs. Their role is to suppress HBV replications by acting on the viral DNA polymerase. Their bioavailability and safety profile is much better than interferon, the first available treatment, which is now much less frequently used.

There are many parameters that are taken into consideration in view of deciding the most appropriate treatment agent. Amongst those are the serum ALT and HBV DNA level, the liver histology, the treatment expense, the patient's age and comorbidities as well as the potential adverse events[4].

In developing countries that there are financial restraints and limited availability, lamivudine and adefovir are the most commonly used first-line therapeutic agents. Interferon (IFN) is of high value as well, as the length of treatment is not more than a year with durable virologic response[4].

Treatment-naïve patients are usually treated with one or more nucleoside analogs with 70-85% success rates after the first year of treatment.

The available oral nucleoside and nucleoside analogs have excellent resistance profiles and lack of side effects. After HBeAg seroconversion, a minimum of 6 months treatment has been recommended by all international liver societies.

In most of the cases, lamivudine is no longer first-line treatment due to high resistance rates. It is still considered as a short-term therapy, especially in patients undergoing chemotherapy as well as in developing countries.

Lamivudine-resistant cases were initially treated with adefovir (potentially nephrotoxic), which has more recently grossly been replaced by tenofovir.

Entecavir is a more potent nucleoside analogue with good benefits of long term use, such as progressive regression of fibrosis, reversal of cirrhosis and decrease HCC incidence.

Telbivudine was found to be more potent than lamivudine but has a very high rate of resistance so had fallen out of favour to be used as monotherapy and can be used as a good alternative to adenofovir/tenofovir in patients with renal impairment.

Emtricitabine is structurally similar to lamivudine but has not been clinically approved.

Interferon is mainly used in pegylated forms (*IFN-a*) as it is better tolerated and has higher antiviral potency. It has been approved by FDA for 48 weeks of treatment, though it has limited use in patients with cirrhosis as flare ups of ALT may occur during therapy. Interferon has not shown drug resistance, unlike nucleoside analogs. **(Table 2)**

Nucleoside analogs and interferon have also shown differences in viral kinetics and gives a rational for combined treatment.

Fortunately, treatment with nucleoside analogs has been shown to be safe and there has been remarkable decline in the need for liver transplantation in patients with cirrhosis[4].

Drug	Dose in Adults*	Use in Children*	Pregnancy Category†	Potential Side Effects†	Monitoring on Treatment‡
Preferred					
Peg-IFN-α-2a (adult) IFN-α-2b (children)	180 mcg weekly	≥1 year dose: 6 million IU/m^2 three times weekly§	C	Flu-like symptoms, fatigue, mood disturbances, cytopenia, autoimmune disorders in adults, anorexia and weight loss in children	Complete blood count (monthly to every 3 months) TSH (every 3 months) Clinical monitoring for autoimmune, ischemic, neuropsychiatric, and infectious complications
Entecavir	0.5 mg daily‖	≥2 years dose: weight-based to 10-30 kg; above 30 kg: 0.5 mg daily‖	C	Lactic acidosis (decompensated cirrhosis only)	Lactic acid levels if there is clinical concern Test for HIV before treatment initiation
Tenofovir dipovoxil fumarate	300 mg daily	≥12 years	B	Nephropathy, Fanconi syndrome, osteomalacia, lactic acidosis	Creatinine clearance at baseline If at risk for renal impairment, creatinine clearance, serum phosphate, urine glucose, and protein at least annually Consider bone density study at baseline and during treatment in patients with history of fracture or risks for osteopenia Lactic acid levels if there is clinical concern Test for HIV before treatment initiation
Tenofovir alafenamide	25 mg daily	—	There are insufficient human data on use during pregnancy to inform a drug-associated risk of birth defects and miscarriage.	Lactic acidosis	Lactic acid levels if clinical concern Assess serum creatinine, serum phosphorus, creatinine clearance, urine glucose, and urine protein before initiating and during therapy in all patients as clinically appropriate Test for HIV before treatment initiation
Nonpreferred					
Lamivudine	100 mg daily	≥2 years dose: 3 mg/kg daily to max 100 mg	C	Pancreatitis Lactic acidosis	Amylase if symptoms are present Lactic acid levels if there is clinical concern Test for HIV before treatment initiation
Adefovir	10 mg daily	≥12 years	C	Acute renal failure Fanconi syndrome Lactic acidosis	Creatinine clearance at baseline If at risk for renal impairment, creatinine clearance, serum phosphate, urine glucose, and urine protein at least annually Consider bone density study at baseline and during treatment in patients with history of fracture or risks for osteopenia Lactic acid levels if clinical concern
Telbivudine	600 mg daily	—	B	Creatine kinase elevation and myopathy Peripheral neuropathy Lactic acidosis	Creatine kinase if symptoms are present Clinical evaluation if symptoms are present Lactic acid levels if there is clinical concern

*Dose adjustments are needed in patients with renal dysfunction.
†In 2015, the U.S. Food and Drug Administration replaced the pregnancy risk designation by letters A, B, C, D, and X with more specific language on pregnancy and lactation. This new labeling is being phased in gradually, and to date only TAF includes these additional data.
‡Per package insert.
§Peg-IFN-α-2a is not approved for children with chronic hepatitis B, but is approved for treatment of chronic hepatitis C. Providers may consider using this drug for children with chronic HBV. The duration of treatment indicated in adults is 48 weeks.
‖Entecavir dose is 1 mg daily if the patient is lamivudine experienced or if they have decompensated cirrhosis.
Abbreviation: TSH, thyroid stimulating hormone.

Table 2: Approved antiviral therapies in adults and children[10].

Response to treatment

When assessing response to treatment we are looking at the biochemical, virologic and histologic response. The desirable results are normalization of ALT levels, disappearance of HBV DNA from serum for at least 6 months after treatment and 2-point or greater improvement in the necroflammatory score without worsening fibrosis respectively.
"Irrespective of the type of antiviral therapy used, the best assurance that late relapses will not occur is provided by the disappearance of HBsAg, with or without seroconversion to anti-HBs; the occurrence comes closest to a clinical cure of hepatitis B."[4].

Monitoring is recommended every 4 months during the first year of treatment and once HBV DNA level is less than 2000 IU/ml or undetectable that can be extended to 6 months.
In case of treatment failure or virologic rise patients should be carefully questioned regarding drug adherence. Drug resistance should of course be considered as well. There are certain mutations that have been identified and could be detected by reverse hybridization assay[4].

Immunoprophylaxis

Immunization can be either passive using HBIG (from plasma with high titers of anti-HBs) or active using inactive HBsAg. The lesser gives long-term immunity.
The currently marketed HBV vaccines use recombinant DNA technology with introduction of the HBsAg gene into a yeast genome. No serious side effects have been reported. 100% protection can be achieved with antibody titers greater than 100 mIU/ml[4]. Vaccination is typically scheduled for months 0, 1, and 6. In case of mucous, percutaneous or ocular exposure, post-exposure vaccination is strongly recommended.

CHAPTER A4: Viral Hepatitis C

Worldwide, more than 200 million people have been infected with hepatitis C virus (HCV)[4]. Globally, it is estimated that 71 million people have chronic HCV, a significant number of which develop cirrhosis or hepatocellular carcinoma (HCC) with an approximate of 399.000 dying each year[7]. In Europe and the United states HCV related complications are the leading indication for liver transplantation[4]. There is no available vaccination currently, though research is ongoing[7].

The only chronic viral infection that can be cured with treatment is HCV[11]. Antiviral treatments can cure more than 95% of the infected individuals[7]. Sustained virological response (SVR), defined as the absence of HCV RNA in serum 3 - 6 months after treatment cessation, is almost always associated with long-lasting eradication of the virus[12,13].

Treatment with IFN-a and ribavirin has accomplished 60-90% SVR on patient infected with genotype 2 and 3 of the virus. Ongoing progress on the studying of the virus and its mechanism of action has led to the development of new therapeutic agents, achieving cure rates above 90%[4].

The HCV is a single stranded enveloped RNA virus with a diameter of 50nm and belongs to the Flaviviridae family, member of the Hepacivirus genus[14]. The virus replicates mainly at the hepatocytes. It attaches to the cell wall and its presumed to enter with endocytosis[4]. It then moves to the endoplasmic reticulum were viral particles are formed with interaction with the genomic RNA. Those are then released and either infect adjacent hepatocytes or enter the circulation[15].

Genotypes and quasispecies

There is great heterogeneity noted at the HCV genome due to the high mutational rate of the virus, as well as the quasispecies generation[4,16].
A high number of virions, up to 10^{12}, are produced per day[17]. It has been estimated that for every 10^4 to 10^5 nucleotides that are copied one error occurs. Every genetic variant is created in a single cell and that may or may not infect the other liver cells, leading to genetic variation in the liver, serum as well as extrahepatic sites. Due to the huge genetic

differences, the classification of the viral sequences includes a genotype and subtype. There are 6 major genotypes and more than 70 subtypes[16]. The HCV genotype does not change over time, thus it only needs to be determined once in an infected person.
There are geographic and racial differences in the distribution and prevalence of HCV genotypes. Genotype 1b is the most prevalent in Europe, followed by 1a, 3 and 2[18]. The distribution of genotypes is changing over time with immigration, as well as with alterations of the viral transmission modes. Response to treatment is an important clinical association with HCV genotype; however HCV genotype does not seem to have an impact on the severity of liver disease[4].

Generation of quasispecies, as already mentioned, is thought to be accounting too to the genetic heterogeneity of HCV. Viral quasispecies are defined as collections of closely related viral genomes subjected to a continuous process of genetic variation[19]. By this mechanism, the virus may be escaping the host's immune response leading to persistent viral infection[20]. The lack of quasispecies has been associated with clearance of the virus, where the existence of multiple quasispecies with viral persistence[21]. Moreover, larger number of quasispecies has been linked with more rapid progression to liver cirrhosis[22].

Incidence and Prevalence

It has been estimated that the origins of HCV are from western and sub-Saharian Africa[23]. There is marked geographic variation at the HCV prevalence with an increase noted in the years between 1990 and 2010[24]. The prevalence was also noted to be higher in males and in African Americans. Injection drug use, blood transfusion or solid organ transplant before 1992, more than 50 lifetime sexual partners as well as coming from a family with below the poverty level income, are amongst the main risk factors for HCV infection[25]. The increased incidence recorder in the beginning of the millennium is expected to decline by 2030[4].

Transmission

The HCV can be transmitted percutaneously, with blood transfusion and needle stick inoculation, and non-percutaneously, with sexual contact and perinatal exposure.
The implementation of HCV blood screening in 1992 has markedly decreased, basically eliminated, the incidence of transfusion acquired HCV infection[4,26].

The major route of HCV infection has always been injection drug use, with a frequency ranging form 57% to 90%[25]. Despite the overlap between risk factors with HBV and HIV infections, the HCV prevalence remains the highest amongst the three.

Transmission rates to health care workers from infected patients range between 0.3% and 4%. There are many risk factors contributing to that, including the type of needle, the depth of injury, the volume of the inoculum, the viral load, the HIV status as well as the adherence or not to universal precautions[27–29]. At the moment, there is still no treatment available for postexposure prophylaxis.

Regarding sexual transmission of HCV, only a 10% to 20% of patients have reported this as their only risk factor[4]. Furthermore, it was shown that in monogamous, heterosexual relationships with avoidance of sexual intercourse during menstruation, as well as anal sex, the risk of transmission is almost zero[30]. High risk sexual practices, though, as well as HIV positivity can increase the incidence of HCV infection[31,32].

The perinatal transmission risk of HCV infection is low in comparison with the high risk of HBV infection. It varies between 5.1% and 6.7%, with two to three times higher incidence in HIV-HCV coinfected patients[33,34]. There is not much convincing evidence regarding differences between vaginal delivery and caesarian section in HCV transmission, though in many authorities elective caesarian section is recommended before membrane rupture[33]. Breastfeeding by mothers infected by HCV is considered to be generally safe[18,35]. Serologic testing for anti-HCV is not recommended though before 18 months of age as this can be acquired passively and persist, leading to diagnostic confusion[4].

Pathogenesis

The pathogenesis of liver damage is mostly immune mediated. It includes an initial innate and subsequent adaptive response. HCV infected patients have variable immune response and although this is inadequate to eradicate the acute infection, it appears to control how strong the persistent infection will be.

Acute Hepatitis C

Acute hepatitis C infection accounts for about 20% of the acute hepatitides, though it is rarely seen in clinical practice, as the majority of cases are asymptomatic.

HCV RNA can be detected in serum one to three weeks after viral transmission, though incubation period may vary between different routes of transmission[36]. Following the initial rapid increase of HCV RNA in serum, ALT increases four to twelve weeks after the initial infection, reaching level of more than ten times of the upper limit of normal. **(Figure 14)** Bilirubin levels increase in serum on the same time[37].

Acute hepatitis C appears to be more severe in patients with alcohol abuse and HBV or HIV coinfection. Parameters that have been noted to increase the risk of chronicity include older age and male gender. Spontaneous clearance has been found to be higher in symptomatic patient during the acute phase. Moreover, there has been found to be genetic association with the outcome of acute hepatitis[38].

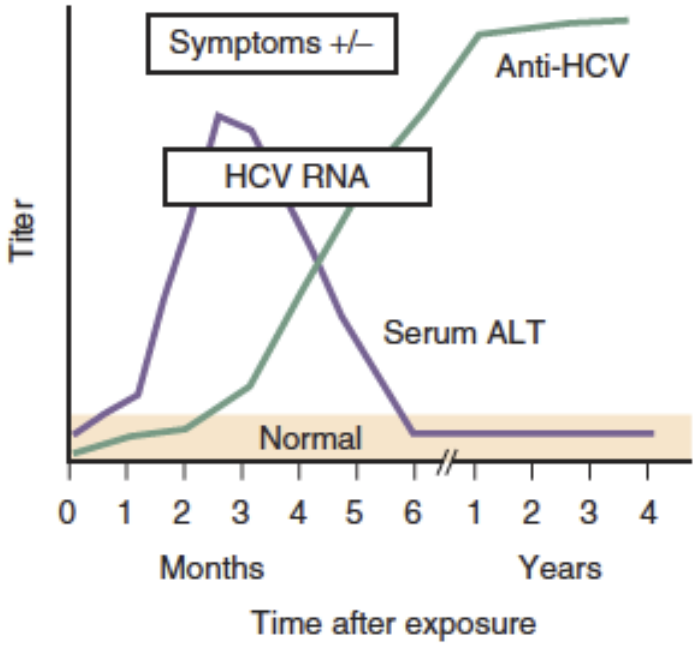

Figure 14: Acute HCV infection course followed by recovery[4].

Chronic Hepatitis C

In chronic hepatitis C serum ALT levels are normally elevated, though they tend to fluctuate, with up to 50% of the patients having a normal ALT level at any given time[39]. This is more common in females and has been associated with lower serum HCV RNA as well as less, histologically proven, hepatic inflammation and fibrosis.

Most patients remain asymptomatic in chronic HCV infection. Symptoms may include myalgias, arthralgias, nausea, sicca syndrome and anorexia.

Extrahepatic manifestations

Extrahepatic manifestations of HCV infection that may occur include amongst others autoimmune thyroiditis, B-cell non-Hodgkin's lymphoma[40], lichen planus, mixed cryoglobulinemia[41], monoclonal gammopathies and porphyria cutanea tarda. These may have an impact on the overall survival[4].

DIAGNOSIS

HCV infection can be detected and monitored with several molecular and immunological assays. Anti-HCV with high serum titers indicates exposure but cannot distinguish between acute, chronic or resolved infection. For diagnosis, serologic assays are used in first instance followed by virologic assays to confirm infection and monitor response to treatment.

Indirect assays

Antibodies against different HCV antigens can be detected with enzyme immunoassays (EIA). There are so far three generation of EIAs. The last generation can detect anti-HCV core antibody as early as seven weeks after infection, with sensitivity and specificity rates reaching 99%[42]. Positive patient should then undergo HCV RNA testing to establish whether they have active viremia[4].

Direct Assays

HCV RNA tests are highly sensitive, quantitative methods and the gold standard for the detection of HCV viremia[43]. Another type of available assays is the transcription-mediated amplifications. Those are extremely sensitive and can detect positivity within one to 3 weeks after infection, though those are not quantitative in the lower range of the test. Unfortunately, the available quantitative tests are non comparable between different assays, so the same laboratory and the same assay should be used throughout antiviral treatment.

The HCV core antigen assay is another alternative method for confirmation of viremia. The advantages are that it is faster and cheaper, though it lacks sensitivity[4].

Genotype

Polymerase Chain Reaction (PCR) and direct sequencing of NS5B or E1 region are the most accurate methods for the identification of HCV genotype. Unfortunately this is not practical in the clinical setting. An alternative method is identifying type-specific antibodies. This has shown to have a 90% concordance when compared with sequence analysis. Other alternative assays include PCR amplification, restriction fragment length polymerase analysis as well as reverse hybridization, which have shown a concordance of 92% to 96%. The most popular commercial assay is a line probe assay called INNO-LiPA. This uses genotype specific probes for reverse transcription of the 5' portion of the HCV genome. Amongst all genotypes, type 1 has the highest identification accuracy.

A negative EIA result is sufficient to exclude HCV infection in low risk patients and a positive EIA is sufficient to confirm the diagnosis if there are elevated serum ALT levels. HCV RNA should be measured if the ALT levels are not elevated[4].

ASSESSMENT OF LIVER FIBROSIS

The liver damage from HCV infection can be variable. Therefore, assessing the degree of hepatic injury is essential. The assessment is typically done by a percutaneous liver biopsy, but noninvasive methods are also available. Thereafter, the hepatic injury can be quantified with several scoring systems.

The first available system was the Histology Activity Index (HAI) or Knodell Score. This grading system combines inflammation and fibrosis, which makes it more insensitive to alterations in fibrosis. It includes periportal inflammation and bridging necrosis, intralobular degeneration and focal necrosis, portal inflammation, and fibrosis. It has a maximum score of 22, with higher scores indicating more advanced disease[45].

The Scheuer system is a simpler scoring that separates inflammation from fibrosis and it has a maximum score of 12[46].

The Ishak system is a modified Knodell's score which stages fibrosis in 6 levels[47]. This enables identification of smaller changes in fibrosis and has made this scoring system more popular, especially in clinical trials. Other available scoring systems include the METAVIR score, the Batts-Ludwing (modified Scheuer) system and the Laennec staging system[48].

Examination of the liver biopsy although important for establishing inflammation and fibrosis, it has its limitations. Those include the associated morbidity and mortality (0.03%), the cost, poor patient tolerance, variability in interpretation as well as sample error[49,50]. Due to these limitations, several noninvasive tests have been developed for the assessment of liver fibrosis. The FibroTest composes a score derived from a_2-macroglobulin, haptoglobin, apolopoprotein A-1, GGTP, and total bilirubin, and it is adjusted for gender and age[51]. Another test is the AST/platelet ratio index (APRI) and is mainly used to exclude or diagnose cirrhosis[52]. Newer techniques such as transient elastography (Fibroscan), acoustic radiation force impulse imaging and magnetic resonance elastography are now available to assess the liver elasticity, which relates with the extent of liver fibrosis[53,54].

The accuracy of predicting fibrosis and cirrhosis, and thus avoiding liver biopsy, can increase with the combination of those techniques with serum markers[55,56]. Moreover, liver biopsy is not deemed essential when there are clinical finding or imaging suggesting cirrhosis[4].

NATURAL HISTORY

In chronic hepatitis C infection there can rarely be spontaneous clearance. HCV can lead to liver damage, subsequently resulting in cirrhosis and hepatocellular cancer. Cirrhosis rates can be variable with a calculated average rate of 16% within 20 years from infection onset[57]. This percentage was shown to differ within regions and from the presence of co-factors[58,59]. Age over forty is very important risk factor, as well as longer duration of infection, alcohol consumption, HBV/HIV coinfection[4]. On the contrary, there seems to be a milder course if the infection if acquired during childhood[60]. In general, regardless of the age of infection, cirrhosis develops in most of the patients by about 65 years of age[61]. Moreover, there possibly seems to be a hormonal protective effect in females as progression to fibrosis was seen to be faster in men[60]. HLA class I and II antigens variants as well as genes polymorphisms have been suggested to have an involvement in fibrosis progression[60–62].

Aminotransferase serum levels are widely used for ongoing monitoring of intrahepatic inflammation. In chronic hepatitis C elevated ALT levels seem to be linked with increased risk of progression to liver fibrosis[61–63].

HCV genotype seems to also play a role in disease progression. Genotype 3 has shown faster disease progression consistent accounting for higher mortality rates[64,65]. Patients infected with HCV genotype 2 seem to experience more frequent flares[66].

Histological feature of chronic hepatitis C is hepatic steatosis, which is further linked to the stage of liver fibrosis[64].

As already known, chronic alcohol consumption in excess is a major cause of liver cirrhosis. On the contrary, consumption of coffee has been reported to be beneficial on the overall course and mortality from HCV[67].

Chronic hepatitis C accounts for about 25% of reported Hepatocellular Carcinoma (HCC) cases worldwide. The prevalence is particularly high in East Asia[68]. In non-cirrhotic patients with advanced liver fibrosis there is a reported 0.8% annual incidence rate, which reaches 1.4-4.9% in patients with cirrhosis[69–71]. The 5-year HCC risk can be as high as 7-30% in HCV related cirrhosis[72,73]. HBV co-infection further increases this risk[4].

TREATMENT

The first approved monotherapy treatment for chronic hepatitis C was IFN-a. Significant advances introduced pegylated longer-acting formulations of interferon as well as the oral analog ribavirin. Telaprevir and boceprevir were the first direct acting antiviral agents (DAAs) approved for treatment of genotype 1 HCV infection in 2011 followed by simeprevir in 2013, which is another protease inhibitor. Revelation of the replication mechanism of HCV has led to further classes of DAAs, such as NS5A inhibitors and nucleoside and non-nucleoside HCV polymerase inhibitors[74]. Ongoing research has been increasingly individualizing treatment based on the HCV genotype, stage of liver disease and availability of DAAs[4.]

On HCV treatment, eradication of the virus is the primary aim and therefore preventing the development of decompensated cirrhosis, HCC and the associated deaths. An excellent marker for the resolution of the hepatitis C infection is the absence of detectable virus in the blood 12-24 weeks after treatment completion, with late relapsing being rare. In particular, long-term follow-up studies have shown that in more than 98% of the cases of patients with negative HCV RNA in serum 24 weeks after treatment completion there was sustained response[12,13]. That was also associated with reduction of hepatic inflammation, regression of fibrosis and subsequent improvement of the quality of life of the successfully

treated patients[75,76]. Antiviral treatment also had reduction of chronic HCV related deaths as well as hepatic decompensation, even in patients with advanced liver fibrosis[77]. In contrary, in patients who did not achieve viral clearance with standard or pegylated IFN treatment, maintenance treatment for 3-4 years with low-dose IFN-a did not show prevention of disease progression[78].

Virological response

During treatment there are more markers of virological response monitored in order to help with treatment guidance. Undetectable HCV RNA in serum after 4 weeks of treatment is defined as rapid virological response (RVR) and is used to identify the most sensitive to treatment patients. RVR was noted in 80-90% HCV genotype 1-infected patients who complete 48 weeks of treatment or HCV genotype 2 or 3 infected patients who complete 24 weeks of antiviral therapy[18]. Moreover, more than 98% of cases have shown failure of treatment in the absence of an early virological response (EVR), which is defined as viral drop of more than 2-log at 12 weeks of therapy. Undetectable HCV RNA at the end of treatment or end-of treatment response (ETR) is used as marker for treatment with ribavirin and pegylated interferon[4].

MEDICATION

Interferons

IFNs are naturally occurring glycoproteins with antiviral, antiproliferative and immunomodulatory effects and have been the cornerstone of HCV antiviral therapy since the 1980s. Pegylated IFNs are IFNs bound to polyethylene glycol (PEG), which enlarges the size of the molecule and increases the IFN half-life allowing once-weekly dosing[4]. Moreover, pegylated IFNs have significantly increases SVR[79].

Ribavirin

Ribavirin, an oral guanosine analog, which acts against DNA and RNA viruses is used in combination with IFN and improves the ETR and relapse rate. It is generally well tolerated, though there is dose dependent hemolytic anemia. It is also teratogenic and patients and

their partners should avoid pregnancy for six months after treatment cessation. Ribavirin is excreted by the kidneys, hence needs to be adjusted for the renal function and used with extreme caution, as it is not removed by hemodialysis[4].

Direct-acting antiviral agents (DAAs)

DAAs inhibit HCV replication mainly by targeting HCV protease, HCV NS5A protein and HCV polymerase. HCV protease inhibitors have high antiviral potency and different levels of resistance. Response rates are shown to be better in HCV genotype 1b rather than in 1a infections[80]. These regimes were developed to be used without interferons[81].

NS5A inhibitors have very high antiviral potency at very small doses. There are limited data in non-genotype 1 HCV patients and the first agent approved by FDA in 2014 was ledipasvir.

Most HCV polymerase inhibitors act mainly against HCV genotype 1b and, to a lesser extent, against genotype 1a. Those are mainly used in combinations with other DAAs.

Acute HCV treatment

Early acute HCV infection treatment is effective in contrast with post-exposure prophylaxis. Many cohort studies have shown more than 85% response rates in early treatment of acute hepatitis C with IFN alone, though as a significant number of patients may clear the infection spontaneously, delayed treatment can be considered[82,83]. IL28B genotype can be used as an indicator of spontaneous recovery, though not solely as the positive predictive value is below 90%[84,85]. Delayed therapy is effective and can definitely considered in patients with IL28B CC genotype[86], but in early treatment fewer patients are lost in follow up[4]. In the context of co-infection with HIV, acute hepatitis C treatment can be similar, though most experts prefer immediate therapy[87]. The role of DAAs is yet to be defined.

Chronic HCV treatment

Interferon dose is the same for all hepatitis C genotypes. Ribavirin dose is based on patient's weight and there are different recommendations depending on the genotype. In HCV genotype 1 and 4 treatment with pegylated IFN-a and ribavirin treatment is administered for 48 weeks where in genotypes 2 or 3 is administered for 24 weeks. In some patients with

genotype 2 or 3 sometimes 12 to 16 weeks of treatment might be enough to achieve SVR. Similarly, in genotype 1 HCV infection with low baseline viral load 24 weeks might be enough[4,18].

Response to treatment can be very well predicted by the rate of the initial fall in HCV RNA levels. Other pretreatment predicting factors for good response include low baseline HCV RNA levels, non-genotype 1 infection, absence of fibrosis or cirrhosis and obesity, lack of insulin resistance or hepatic steatosis, absence of HIV infection and white race. The most powerful predicting factor though is the HCV genotype[4.]

Protease inhibitors have the advantages of better tolerability and can be used in IFN-free or IFN-sparing regimes.

It is possible to cure HCV infection. In vitro and in small animal models this has been achieved with combination of DAAs that target different steps in hepatitis C virus life cycle[87,88]. More IFN-free regimes are being explored and it is likely that genotype 1 will be the most easy to cure. Genotype 2 will be also cured with newer therapies, though genotype 3 will possibly remain a challenge.

Increasingly more patients have become candidates for treatment. Physicians who are not comfortable administering and monitoring the treatment therapy should be referring to experienced gastroenterologists and hepatologists[35]. Treatment is best to be initiated before liver cirrhosis develops, as there are better response rates and can offer reduction to later complications of cirrhosis.

There are few absolute contraindications to treatment that remain. These include adequate contraception during and up to 6 months after treatment for the use of ribavirin as it is teratogenic, psychiatric conditions that are severe or uncontrolled and severe pulmonary or cardiac disease; there is no survival benefit in patients with severe comorbidities.

Baseline investigations prior initiation of treatment includes liver function tests, full blood count and thyroid-stimulating hormone (TSH) levels. Women in childbearing age require a negative pregnancy test before initiation of treatment with ribavirin. Moreover, HCV RNA levels and genotype will determine the duration of therapy and the dose of ribavirin[4].

The majority of the haematologic side effects, and in particular haemolytic anaemia, will occur within the first month after initiation of treatment including IFN and ribavirin and, thus, full blood counts need to be performed on a weekly basis. After the first month, monthly full blood counts and serum ALT levels should be checked and three-monthly TSH levels. Serum HCV RNA levels are essential to determine the duration of treatment and should be performed at baseline, 4 weeks, 12 weeks and if still detectable at 24 weeks. On achievement of SVR, annual testing is recommended for up to two years. For relapsers or nonresponders the recommendations suggests annual laboratory testing and check-ups, as well as repeat liver biopsy every four to five years[4].

Most frequent reasons for dose reduction are anaemia, neutropenia and thrombocytopenia. When IFN is used more than 90% of the patients experience flu-like symptoms and 10% to 30% alopecia. Most common side effects with ribavirin are anaemia, pharyngitis, cough, dyspnea, insomnia, rash, pruritus and nausea. Protease inhibitors can interact with many drugs as they inhibit cytochrome P450. At large, the newer oral regimens have milder and well-tolerated side effects[4].

Table 3

Regimen	Patient Population	Duration (Weeks)	Caveats and Other Considerations
Genotype 1			
Daclatasvir + sofosbuvir	Decompensated cirrhosis regardless of subtype	12	Add dose-escalating RBV[a]
	HIV/HCV coinfection when antiretroviral regimen cannot be made to accommodate recommended regimens	12	
Elbasvir/grazoprevir	Treatment naive or PEG/RBV experienced regardless of cirrhosis	12	For GT1a, check RASs to NS5A; use a different recommended regimen if high-fold variants detected
	Severe renal impairment (CKD stage 4/5)	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Glecaprevir/pibrentasvir	Treatment naive or PEG/RBV experienced without cirrhosis	8	
	Treatment naive or PEG/RBV experienced with cirrhosis, and non-NS5A failures (including NS3) regardless of cirrhosis	12	
	Post liver transplant without cirrhosis	12	
	Severe renal impairment (CKD stage 4 or 5)	8–12	Treatment duration depends on presence of cirrhosis
	Post kidney transplant regardless of cirrhosis	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Ledipasvir/sofosbuvir	Treatment naive regardless of cirrhosis	12	
	Treatment naive, no cirrhosis, non-black, HIV negative, and HCV RNA $<10^6$ IU/mL	8	
	PEG/RBV (± NS3 protease inhibitor) experienced without cirrhosis	12	
	Decompensated cirrhosis, treatment naive or PEG/RBV (± NS3 protease inhibitor) experienced	12	Add dose-escalating RBV[a]
	Decompensated cirrhosis, prior sofosbuvir failure only	24	Add RBV
	Post liver transplant regardless of cirrhosis or decompensation	12	Add dose-escalating RBV[a]
	Post kidney transplant regardless of cirrhosis	12	
Sofosbuvir/velpatasvir	Treatment naive or PEG/RBV ± NS3 protease inhibitor experienced regardless of cirrhosis	12	Same for GT1a and GT1b
	GT1b, non-NS5A DAA experienced regardless of cirrhosis	12	
	Decompensated cirrhosis, treatment naive or PEG/RBV (± NS3 protease inhibitor) experienced	12	Add dose-escalating RBV[a]
	Decompensated cirrhosis, DAA failure (including NS5A)[b]	24	Add RBV
Sofosbuvir/velpatasvir/voxilaprevir	NS5A failures (including NS3 protease inhibitor) regardless of cirrhosis	12	Same for GT1a and GT1b
	GT1a, non-NS5A failures (including NS3 protease inhibitors) regardless of cirrhosis	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Genotype 2			
Daclatasvir + sofosbuvir	Decompensated cirrhosis[b]	12	Add dose-escalating RBV[a]
	Post liver transplant regardless of cirrhosis or decompensation[b]	12	Add dose-escalating RBV[a]
Glecaprevir/pibrentasvir	Treatment naive or PEG/RBV experienced without cirrhosis	8	
	Treatment naive or PEG/RBV experienced with cirrhosis, and sofosbuvir failures regardless of cirrhosis	12	
	Post liver transplant without cirrhosis	12	
	Severe renal impairment (CKD stage 4 or 5)	8–12	Treatment duration depends on presence of cirrhosis
	Post kidney transplant regardless of cirrhosis	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		

Table 3 continued

Regimen	Patient Population	Duration (Weeks)	Caveats and Other Considerations
Sofosbuvir/velpatasvir	Treatment naive, or PEG/RBV or non-NS5A experienced regardless of cirrhosis	12	
	Decompensated cirrhosis, treatment naive or PEG/RBV or non-NS5A experienced	12	Add weight-based RBV
	Decompensated cirrhosis, DAA failure (including sofosbuvir ± NS5A)[b]	24	Add dose-escalating RBV[a]
	Post liver transplant with decompensated cirrhosis	12	Add weight-based RBV
Sofosbuvir/velpatasvir/voxilaprevir	NS5A failures	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Genotype 3			
Daclatasvir + sofosbuvir	Decompensated cirrhosis	12	Add dose-escalating RBV[a]
	Post liver transplant regardless of cirrhosis or decompensation	12	Add dose-escalating RBV
Glecaprevir/pibrentasvir	Treatment naive without cirrhosis	8	
	Treatment naive with compensated cirrhosis	12	
	Post liver transplant without cirrhosis	12	
	Severe renal impairment (CKD stage 4 or 5)	8–12	Treatment duration depends on presence of cirrhosis
	Post kidney transplant regardless of cirrhosis	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Sofosbuvir + elbasvir/grazoprevir	PEG/RBV experienced with compensated cirrhosis[b]	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Sofosbuvir/velpatasvir	Treatment naive without cirrhosis	12	
	Treatment naive with cirrhosis or PEG/RBV experienced without cirrhosis	12	Check for Y93H RAS; if present, use a different recommended regimen when available or 12 weeks of sofosbuvir/velpatasvir/voxilaprevir (an alternative regimen)[b]
	Decompensated cirrhosis, treatment naive or PEG/RBV experienced	12	Add weight-based RBV[a]
	Decompensated cirrhosis, previously exposed to DAA (including sofosbuvir ± NS5A)[b]	24	Add weight-based RBV
	Post liver transplant with decompensated cirrhosis	12	Add weight-based RBV
Sofosbuvir/velpatasvir/voxilaprevir	PEG/RBV experienced with cirrhosis, or DAA failure (including NS5A inhibitors) regardless of cirrhosis	12	Add RBV for prior NS5A inhibitor failure and cirrhosis[b]
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Genotype 4			
Daclatasvir + sofosbuvir	Decompensated cirrhosis[b]	12	Add dose-escalating RBV[a]
	HIV/HCV coinfection when antiretroviral regimen cannot be made to accommodate recommended regimens	12	
Elbasvir/grazoprevir	Treatment naive or PEG/RBV experienced with prior relapse, regardless of cirrhosis	12	Not recommended for other treatment failures
	Severe renal impairment (CKD stage 4/5)	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Glecaprevir/pibrentasvir	Treatment naive or PEG/RBV experienced without cirrhosis	8	
	Treatment naive or PEG/RBV experienced with cirrhosis	12	
	Post liver transplant without cirrhosis	12	
	Severe renal impairment (CKD stage 4 or 5)	8–12	Treatment duration depends on presence of cirrhosis
	Post kidney transplant regardless of cirrhosis	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		

Table 3 continued

Regimen	Patient Population	Duration (Weeks)	Caveats and Other Considerations
Ledipasvir/sofosbuvir	Treatment naive regardless of cirrhosis or PEG/RBV experienced without cirrhosis	12	
	Decompensated cirrhosis, treatment naive or PEG/RBV experienced	12	Add dose-escalating RBV[a]
	Decompensated cirrhosis, sofosbuvir failure[b]	24	Add weight-based RBV
	Post liver transplant regardless of cirrhosis or decompensation	12	Add dose-escalating RBV[a]
	Post kidney transplant regardless of cirrhosis	12	
Sofosbuvir/velpatasvir	Treatment naive or PEG/RBV experienced regardless of cirrhosis	12	
	Decompensated cirrhosis, treatment naive or PEG/RBV (± NS3 protease inhibitor) experienced	12	Add weight-based RBV[a]
	Decompensated cirrhosis, DAA failure (including NS5A)[b]	24	Add weight-based RBV
Sofosbuvir/velpatasvir/voxilaprevir	NS5A failures (including NS3 protease inhibitors) regardless of cirrhosis	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Genotype 5 or 6			
Glecaprevir/pibrentasvir	Treatment naive or PEG/RBV experienced without cirrhosis	8	
	Treatment naive or PEG/RBV experienced with cirrhosis	12	
	Post liver transplant without cirrhosis	12	
	Severe renal impairment (CKD stage 4 or 5)	8–12	Treatment duration depends on presence of cirrhosis
	Post kidney transplant regardless of cirrhosis	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		
Ledipasvir/sofosbuvir	Treatment naive or PEG/RBV experienced regardless of cirrhosis	12	
	Decompensated cirrhosis, treatment naive or PEG/RBV experienced	12	Add dose-escalating RBV[a]
	Decompensated cirrhosis, sofosbuvir failure[b]	24	Add dose-escalating RBV[a]
	Post liver transplant regardless of cirrhosis or decompensation	12	Add weight-based RBV[a]; use dose-escalating RBV if decompensated
Sofosbuvir/velpatasvir	Treatment naive or PEG/RBV experienced regardless of cirrhosis	12	
	Decompensated cirrhosis, treatment naive or PEG/RBV (± NS3 protease inhibitor) experienced	12	Add weight-based RBV[a]
	Decompensated cirrhosis, DAA failure (including NS5A)[b]	24	Add weight-based RBV
Sofosbuvir/velpatasvir/voxilaprevir	NS5A failures (including NS3 protease inhibitors) regardless of cirrhosis	12	
	Not for decompensated cirrhosis or post liver transplant with cirrhosis		

Cirrhosis refers to compensated cirrhosis unless otherwise specified.

Abbreviations: CKD, chronic kidney disease; DAA, direct-acting antiviral agent; GT, genotype; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NS5A, nonstructural protein 5A; PEG, peginterferon; RAS, resistance-associated substitution; RBV, ribavirin.

[a]Extend treatment duration to 24 weeks if RBV ineligible.

[b]Represents off-label use.

Table 3: Summary of treatment recommendations for initial and re-treatment of HCV genotype 1-6 infection[89].

CHAPTER A5: Liver Fibrosis and Cirrhosis

Hepatic cirrhosis is defined as replacement of normal liver architecture by fibrous tissue in the form of abnormal nodules. It is the end result of chronic liver diseases and most commonly results from chronic hepatitis C and B[90].

Pathogenesis

Hepatic stellate cells are the cells mostly implicated in the pathogenesis of liver fibrosis. Once activated, these transform into myofibroblasts[91]. **(Figure 15)** The activation pathways that are mostly involved in this activation are pathways mediated through transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF) and integrin signaling pathways.

Other cell types that are important in the fibrosis process include epithelial cells, the injury of which culminates in the recruitment and activation of the hepatic stellate cells[92]. Macrophages are also deemed to play an important role in fibrosis as well as angiogenesis[4].

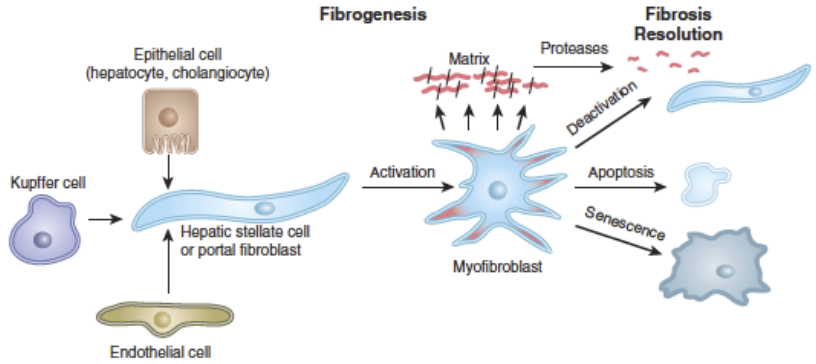

Figure 15: Fibrogenesis schematic overview[4].

Diagnosis

Strictly speaking, cirrhosis is a histological diagnosis, though the diagnosis can be a result of a combination of clinical, imaging and laboratory findings.

Physical findings that are suggestive of cirrhosis include palmar erythema, terry's nails, clubbing of the fingernails, gynecomastia, spider telangiectasias, dilated abdominal veins (caput medusa) and hepatosplenomegaly. Those are mainly resulting from the alteration in the metabolism of estrogens by the cirrhotic liver and portal hypertension. In patients with

ascites, eosophageal varices or hepatic encephalopathy on a background of chronic liver disease, liver biopsy is not essential as they are likely to have cirrhosis. Laboratory findings that can suggest cirrhosis include deranged liver function tests, hypoalbuminemia and prolongation of prothrombin time. Scoring systems commonly used to outline fibrosis are a serum AST/platelet radio index (APRI), which, if greater than 2 is suggestive of cirrhosis, as does a Bonacini cirrhosis discriminant score (CDS: platelet score + ALT/AST ratio score + INR score) of greater than 7.

Imaging studies such as transient elastography, acoustic radiation force impulse (ARFI) and magnetic resonance elastography (MRE) can aid the diagnosis of cirrhosis if physical and laboratory findings are not suggestive. On transient elastography, liver stiffness is measured in kilopascals and values greater than 14kPa are suggestive of cirrhosis, and greater than 21kPa are associated with portal hypertension[92]. In ARFI imaging, which is more easily performed in comparison with transient elastography, values greater than 2.6 m/sec also suggest cirrhosis[93]. Values greater than 5.9kPa on MRE are suggestive of hepatic cirrhosis and a biopsy is not usually required for confirmation[4].

Natural History

Cirrhosis is largely classified as compensated or decompensated. The latter is characterized by the presence of complications such as ascites, variceal hemorrhage, jaundice, encephalopathy or hepatocellular carcinoma.

Clinically, 4 stages of cirrhosis have been described. In stage 1 there is absence of ascites and varices; in stage 2 there is presence of non-bleeding varices; in stage 3 there is ascites with or without the presence of oesophageal varices; and in stage 4 there is variceal bleeding with or without the presence of ascites[4].

In liver cirrhosis most deaths occur due to decompensation. However, in compensated cirrhosis cardiovascular disease is the most common cause of death[94].

Prognosis

Cirrhosis has an increased risk of death in comparison with the general population, which can be up to 5-fold for compensated cirrhosis and 10-fold for decompensated cirrhosis. In regards to the median survival, that is nine to twelve years in patients with compensated cirrhosis and two in those with decompensated cirrhosis[4].

The prognosis, though, does also depend from the existence of comorbidities. Mortality risk can be calculated with generic scores such as the CHILD-Turcotte-Pugh score and the MELD score. Von Willebrand factor levels have also been associated with higher risk of decompensation and thus mortality[95].

Compensated cirrhosis can progress to decompensated with a 10-year probability of 58%[4]. The annual rate, though, varies depending on the etiology, with a 4% rate for HCV-related cirrhosis and 10% in HBV-related cirrhosis[96].

Management

Surveillance for HCC with 6 monthly ultrasound of the liver is part of the management of compensated cirrhosis, as well as gastrointestinal endoscopy for esophageal varicies and lifestyle changes, with a questioned cost-effectiveness though[97]. The management also includes immunization against HAV, HBV, influenza and pneumococcal pneumonia[4], as well as the use of molecular weight heparin which has been reported to delay decompensation regardless if there is or not portal vein thrombosis[98].

Cirrhotic patients have protein-calorie malnutrition and is essential that to be addressed and is recommended to monitor fat-soluble vitamins and zinc levels. Other problems associated with cirrhosis include fatigue, muscle cramps and sexual dysfunction. Depression occurs in up to 40% of the patients and is associated with diabetes mellitus, obesity and sleep disorders, all of which should be addressed and managed as required[4].

Reversal of fibrosis

Clinical observation and experimental studies in animal models have shown evidence that fibrosis is reversible. Genetic disruption of fibrogenic signaling pathways in animal models has been shown to reverse or prevent liver fibrosis[99]. In humans, though, there are a number of limitations that have precluded successful antifibrotic therapy, including the lack of effective tools to assess fibrosis noninvasively[100]. Moreover, trial design is further complicated by the fact that fibrosis is multifactorial and the resolution might take years to be achieved. Finally, advanced stages of fibrosis might not be amendable to reverse due to fixed angioarchitectural changes[4].

CHAPTER A6: Evaluation of Liver fibrosis

The gold standard for diagnosing cirrhosis has long been liver biopsy. This may, though, be associated with costs and, infrequently, with risks. Moreover, things that must be taken into consideration include sampling error and inter-observer disagreement in the estimation of fibrosis extent[4].

6.1. **Histological grading and staging of fibrosis**

The extend of fibrosis in the liver is one of the most significant and valuable markers for the diagnosis and prognosis of chronic liver disease. It is for that matter considered as a "gold standard"[101]. Evidence has shown that treatment could alter the fibrosis in the liver[101,102]. That makes re-assessment of the degree of fibrosis even more crucial.

Several histological features should be evaluated for the staging of fibrosis. Those include assessment of the extent of the extracellular matrix deposit, the localization of the deposits within the liver lobule and changes in lobular architecture, which are then integrated into a semiquantitative scoring system[103]. **(Figure 16)**

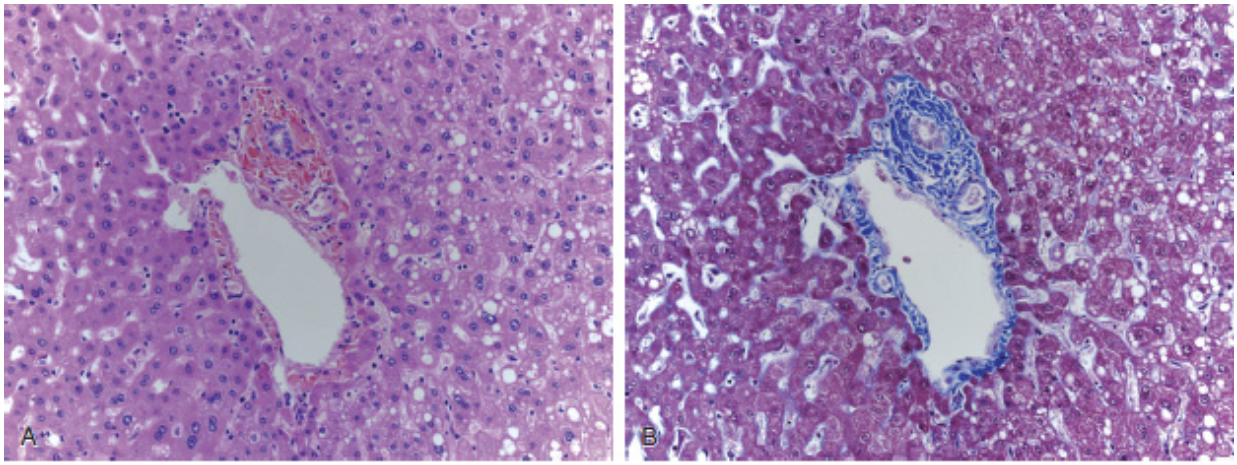

Figure 16: Histologic stages of hepatic fibrosis. A: Stage 0 fibrosis - H&E. Normal portal tract containing a portal vein branch, hepatic artery branch and intralobular bile duct. The parenchyma shows mild steatosis. B: Stage 0 - Masson's trichrome stain. Minimal (normal) amount of collagen in portal tract highlighted in blue.

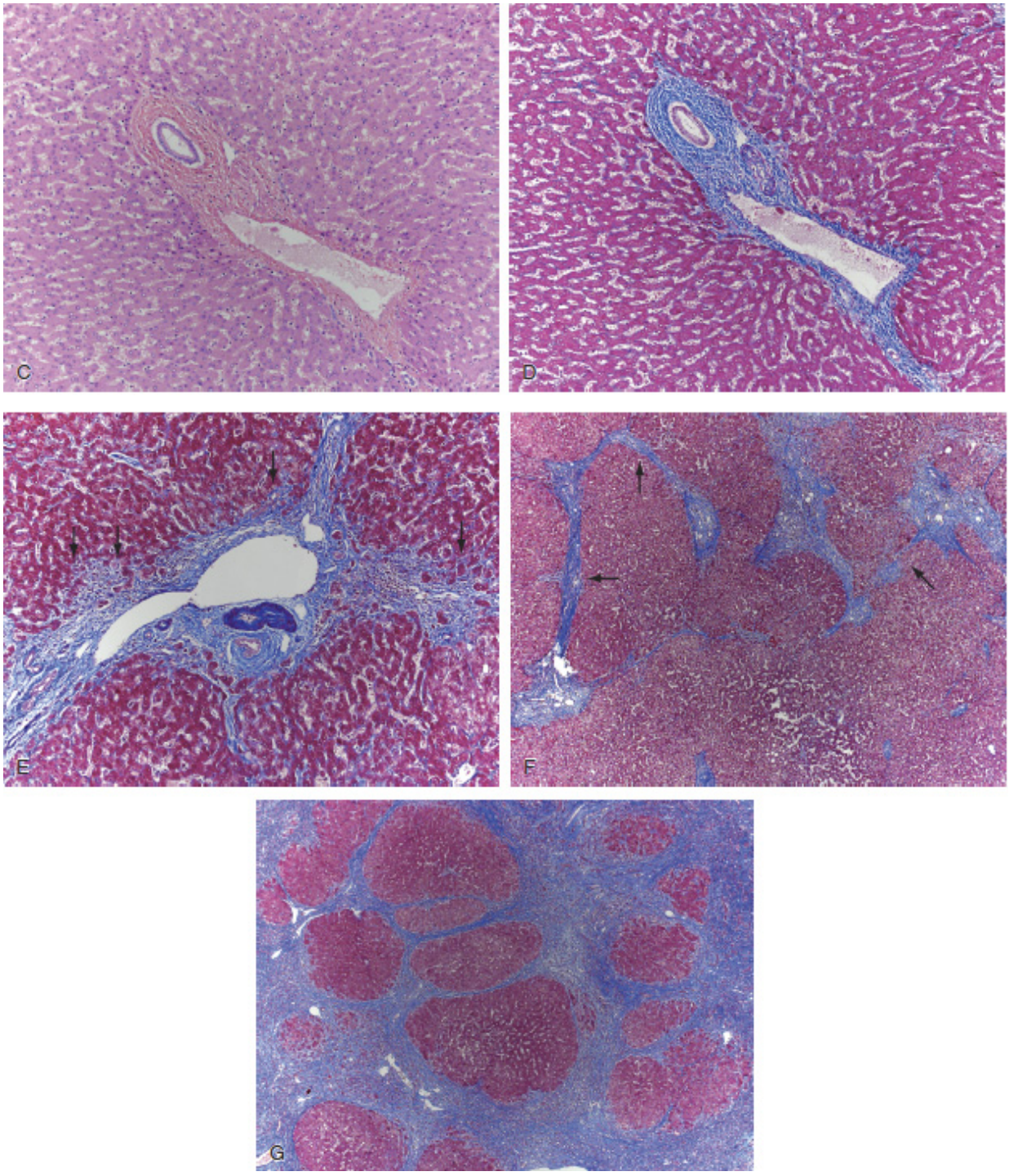

Figure 16 cont'd: C: Stage 1 – H&E. Significant increase of collagen in portal tract. D: Stage 1 – Masson's trichrome stain. Fibrosis expands the portal tract but does not involve the surrounding parenchyma. E: Stage 2 - Masson's trichrome stain. Expansion of the portal tract by fibrosis, which also extends to the surrounding periportal parenchyma (arrows). F: Stage 3- Masson's trichrome stain. Bridging fibrosis is seen (arrows). G: Stage 4 (cirrhosis) - Masson's trichrome stain. Completely distorted normal liver architecture and replaced by regenerative nodules separated by fibrous septa in blue[4].

There are many systems that evaluate the grade (categories of inflammation) and the stage (architectural disruption and fibrosis), such as the Knodell Histology Activity Index (HAI)[44], b) the Ishak HAI[46], c) the Metavir scoring system[47], and d) the Scheuer HAI[45], but their final score share analogous features[101].

Knodell's score or "histological activity index" system (HAI) dates back in 1981. It consists of four histological features: periportal and bridging necrosis, intralobular degeneration - focal necrosis, portal inflammation and fibrosis. **(Table 4)** In this score, Knodell and colleagues excluded from their system on each of the scoring features the number "2" with the purpose of amplifying the difference between mild and severe disease[44,101].

I. Periportal ± bridging necrosis	Score	II. Intralobular degeneration and focal necrosis[b]	Score	III. Portal inflammation	Score	IV. Fibrosis	Score
A. None	0	A. None	0	A. No portal inflammation	0	A. No fibrosis	0
B. Mild piecemeal necrosis	1	B. Mild (acidophilic bodies, ballooning degeneration and/or scattered foci of hepatocellular necrosis in <1/3 of lobules or nodules)	1	B. Mild (sprinkling of inflammatory cells in <1/3 of portal tracts)	1	B. Fibrous portal expansion	1
C. Moderate piecemeal necrosis (involves less than 50% of the circumference of most portal tracts)	3	C. Moderate (involvement of 1/3–2/3 of lobules or nodules)	3	C. Moderate (increased inflammatory cells in 1/3–2/3 of portal tracts)	3	C. Bridging fibrosis (portal–portal or portal–central linkage)	3
D. Marked piecemeal necrosis (involves more than 50% of the circumference of most portal tracts)	4	D. Marked (involvement of >2/3 of lobules or nodules)	4	D. Marked (dense packing of inflammatory cells in >2/3 of portal tracts)	4	D. Cirrhosis[c]	4
E. Moderate piecemeal necrosis plus bridging necrosis[d]	5						
F. Marked piecemeal necrosis plus bridging necrosis[d]							
G. Multilobular necrosis[e]	10						

[a] HAI score is the combined scores for necrosis, inflammation, and fibrosis.
[b] Degeneration: acidophilic bodies, ballooning; focal necrosis: scattered foci of hepatocellular necrosis.
[c] Loss of normal hepatic lobular architecture with fibrous septae separating and surrounding nodules.
[d] Bridging is defined as ≥2 bridges in the liver biopsy specimen; no distinction is made between portal–portal and portal–central linkage
[e] Two or more contiguous lobules with panlobular necrosis.

Table 4: HAI for numerical scoring of liver biopsy specimens[104].

Currently, the most commonly used system is the Ishak (or "revised Knodell") system. This system reintroduces the number "2" in order to alter the previous criticism of the numerical discontinuity[46,101]. This system descriptive strength is potentially more discriminant as it evaluates fibrosis in seven categories[46]. **(Table 5)**

	Score
A. Periportal or periseptal interface hepatitis (piecemeal necrosis)	
Absent	0
Mild (focal, few portal areas)	1
Mild/moderate (focal, most portal areas)	2
Moderate (continuous around <50% of tracts or septa)	3
Severe (continuous around >50% of tracts or septa)	4
B. Confluent necrosis	
Absent	0
Focal confluent necrosis	1
Zone 3 necrosis in some areas	2
Zone 3 necrosis in most areas	3
Zone 3 necrosis+occasional portal-central (P-C) bridging	4
Zone 3 necrosis+multiple P-C bridging	5
Panacinar or multiacinar necrosis	6
C. Focal (spotty) lytic necrosis, apoptosis and focal inflammation*	
Absent	0
One focus or less per 10×objective	1
Two to four foci per 10×objective	2
Five to ten foci per 10×objective	3
More than ten foci per 10×objective	4
D. Portal inflammation	
None	0
Mild, some or all portal areas	1
Moderate, some or all portal areas	2
Moderate/marked, all portal areas	3
Marked, all portal areas	4

*Does not include diffuse sinusoidal infiltration by inflammatory cells.

Maximum possible score for grading	18

Additional features which should be noted but not scored:
Bile-duct inflammation and damage
Lymphoid follicles
Steatosis, mild, moderate or marked
Hepatocellular dysplasia, large- or small-cell
Adenomatous hyperplasia
Iron or copper overload
Intracellular inclusions (e.g. PAS-positive globules, Mallory bodies)

Table 5: Modified HAI grading: necroinflammatory scores[46].

METAVIR scoring system was specifically designed for the evaluation of HCV related chronic hepatitis. The score assesses piecemeal and lobular necrosis, excluding portal inflammation from the algorithm, as this is a criterion on the definition of chronic hepatitis[47,101].

(Figure 17)

Scheuer system is similar to the METAVIR staging system. **(Figure 18)**

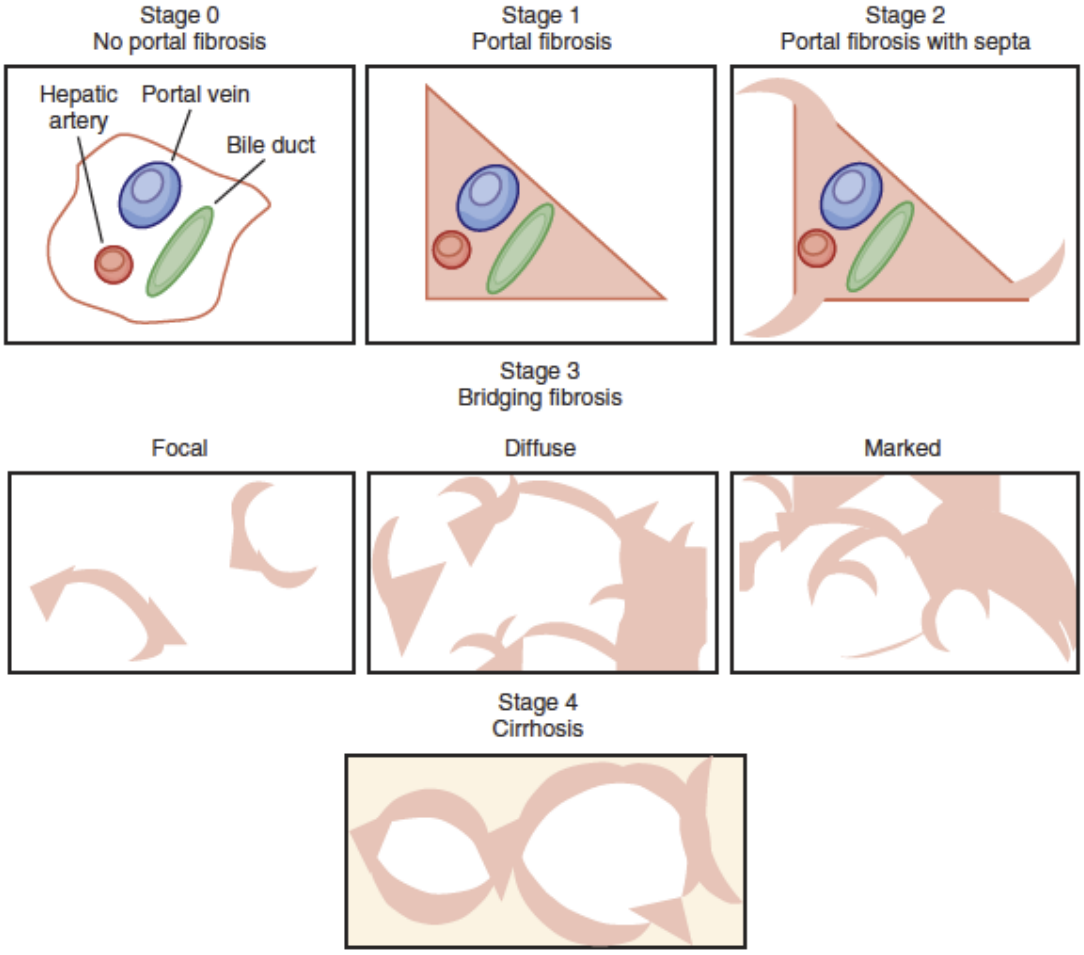

Figure 17: Visual depictions of fibrosis staging (METAVIR system) in chronic hepatitis C. Diagnosis of cirrhosis requires the presence of nodules formation[4].

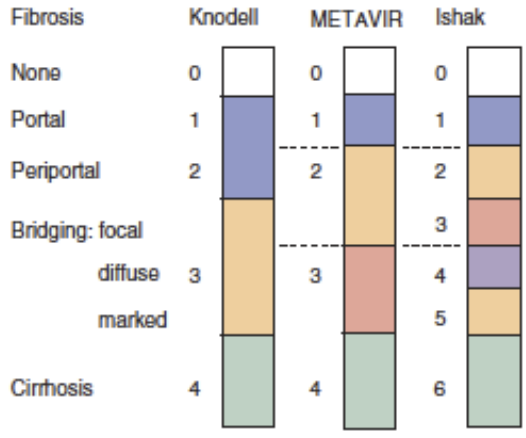

Figure 18: Comparison of the Knodell, METAVIR and Ishak staging systems. The METAVIR system is similar to the Scheuer[4].

Although scoring systems haven been designed and stated to be "numerical, objective and reproducible"[44], observer variability has been well-documented[105–107]. The level of experience of a histopathologist, including the specialization, duration and location of the practice, seemed to have more influence than the specimen characteristics[108]. Organizing consensus meetings after the independent blinded assessment of at least two histopathologists, is a way to attempt and reduce this variability[109].
The size of the biopsy is another important factor that can influence the interpretation, as smaller size is more likely to lower the grade and stage[110]. Reliable is considered a biopsy with a length of 20mm or more, with at least 11 entire portal tracts[110], in order to minimize "underestimation" and "sampling error"[112,113].

In regards to staining, pathologists normally perform several different stainings to assess fibrosis. Those include Masson trichrome, Sirius red, Victoria blue and orcein staining. Masson trichrome and Sirius red are reported to be more specific for collagen staining[111,112]. Sirus red is the preferred one for better quantification of liver fibrosis with techniques like computer assisted digital image analysis (DIA)[115].

6.2. **Automatic quantification of liver fibrosis**

Liver fibrosis can be morphologically measured in histologically stained sections with computer assisted image analysis (IA). With the use of digital images in multiple segmentations, the areas of collagen and tissue are measured in order to produce the collagen proportionate area (CPA), which gives a ratio of fibrosis[101].

Staining plays an important role in enabling histological quantification of hepatic collagen. As already mentioned, Sirius red was recommended because of the good affinity for the majority of the liver collagens including hepatic collagens I and III, which are the most common, as well as the suitability for polarization microscopy[101,116].

Normal human liver collagen has been estimated to about 5.5 mg/g and cirrhotic about 30mg/g[117]. Image analysis studies have looked at fibrosis in several conditions giving similar results for fibrous tissue varying between 1-4% in normal liver and 15-35% in cirrhotic liver[118–121].

Correlation with categorical scores has also been looked at, and variable correlations have been reported[118,120,122]. This was deemed to be due to the fact that categorical scores depend more on architectural changes rather than the amount of fibrous tissue[101]. Despite that, IA measurements have been shown by O'Brien *et al* to correlate well with Ishak's staging score[123].

In bibliography, the methodologies that have been proposed so far have all been semi-automated. All include three stages: image segmentation for tissue background separation, identification of collagen within the liver tissue and removal of all the other tissues, though one cannot find too many details in regards to image analysis techniques[120,122]. Moreover, the cost of these techniques in combination with the lack of specialized tools for digital image analysis, have been the main reasons why these are not yet being used in clinical practice. Nevertheless, more sophisticated techniques are being developed with time, which should bring these advances in daily medical management.

CHAPTER A7: AIM OF THE STUDY

Chronic liver disease is histologically most commonly associated with fibrosis[131]. Determining early and accurately the degree of fibrosis is crucial for diagnosing as well as evaluating the therapeutic efficacy[124]. Patients with chronic hepatitis B and C with deranged liver function tests frequently undergo liver biopsy in order for the amount of fibrosis to be determined. Image analysis (IA) for collagen proportionate area (CPA) assessment of hepatic biopsies got increased attention over the last decade. For appropriate quantification of the collagen, needle biopsy specimens are segmented and stained with special dyes. Ideally, only hepatic tissue should be examined, though, unfortunately, biopsy samples contain other tissues as well, such us structural collagen, capsule collagen, muscles, blood vessels, fat, as well as artifacts, making the use of sophisticated IA techniques extremely valuable.

Aims of our study were to quantitatively assess the amount of liver fibrosis in liver biopsies from patients with hepatitis C before commencing on any treatment and to determine the correlation with Ishak's staging score in view of developing a software that could automatically calculate the stage of fibrosis (Fibrosis Detector). With the use of digital image analysis of histological images and machine learning, the goal was to create a marker of collagen ratio, the Collagen Proportionate Area or CPA.

B. METHODS AND RESULTS

CHAPTER B1: Patient Cohort

The dataset used to evaluate the proposed methodology included 79 images of liver biopsies of patients with HCV from Royal Free Hospital in London. The biopsies were processed with formalin fixation, paraffin embedment and staining with Picrosirius red dye. A Canon Powershot A640 digital camera was used for the photography of the samples. This was connected to a close-up copystand with black-lighting. Every single sample was fitted in a solitary low-resolution image, which was then extracted in a JPEG format. **(Figure 19)** Low resolution allows for quick and robust processing of the images. The images sizing was varying between 337x1001x3 and 1439x1909x3 pixels. RGB color model was used with 24 BitBepth and File size ranging from 104 to 969 KB.

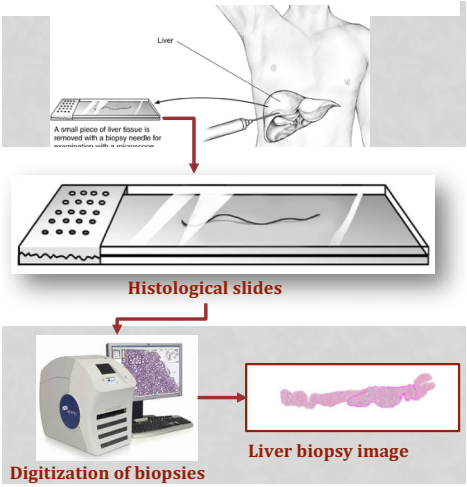

Figure 19: Methodology illustration

The biopsies used were from two different needle sizes. In almost all images (94.94%) one or more large tissue specimens were obtained. This was deemed as sufficient by histopathologists. In the rest 5.06% of images, segmented parts of tissues were presented. The existence of other tissue types, such as structural collagen, muscle and blood, within the samples made the CPA measurement more perplexing and challenging.

We interpreted all the images. Image regions were divided in seven categories: liver tissue, structural collagen, muscular tissue, blood clots, dye stains, artifacts and non-evaluable liver tissue (small fragments). **(Figure 20)** The feature vectors of each region were binary

annotated as liver and non-liver tissue. The binary annotation was based on whether the detected regions were associated or not with an image region. If one or more detected regions were correlating with one image region, then the appropriate labeling followed this and if a detected region was not correlating with any image region, then this was excluded from the study. It would be important to mention though that no liver tissue image falls in our study under the second category. With the use of K-mean (KM) algorithm in the first stage 513 number of patterns were identified, 390 liver and 123 non-liver tissue, whereas there were 515 with the Fuzzy C-means (FCM) algorithm, 392 liver and 123 non-liver tissue. The above difference was attributed to a small variation in pixel segmentation in stage one, which resulted to some regions to be considered connected or not after the use of the clustering algorithm. Lastly, CPA was assessed for each image again.

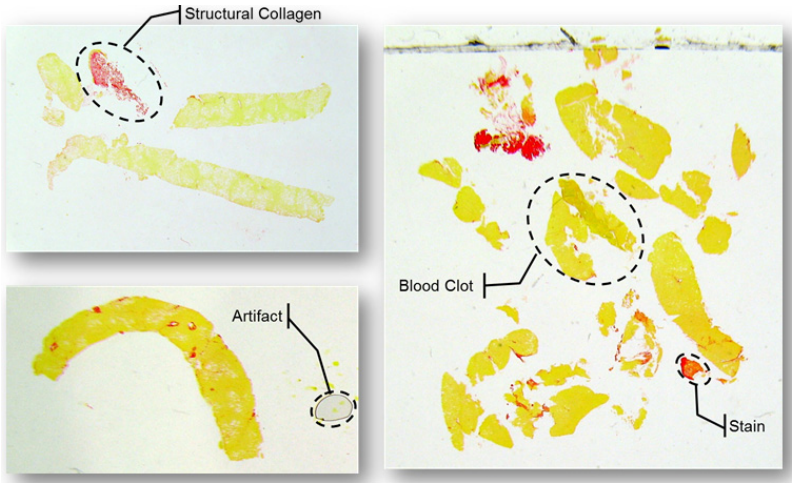

Figure 20: Image regions categories illustration

CHAPTER B2: Study Protocol

We proposed and performed a three-stage methodology for this study. In stage one, background pixels were separated as zero background from pixels belonging to all the other image regions. In stage two, shape and color features were extracted from each region. These features where then used for classification and identification of each region and where stratified as liver or non-liver tissue. The non-liver tissue regions were then rejected and the liver tissue regions undergone further processing with CPA computation, which was the third stage. At this final stage, clustering algorithms were used to further divide pixels into two groups; the fibrosis pixels and the normal liver tissue pixels. **(Figure 21)**

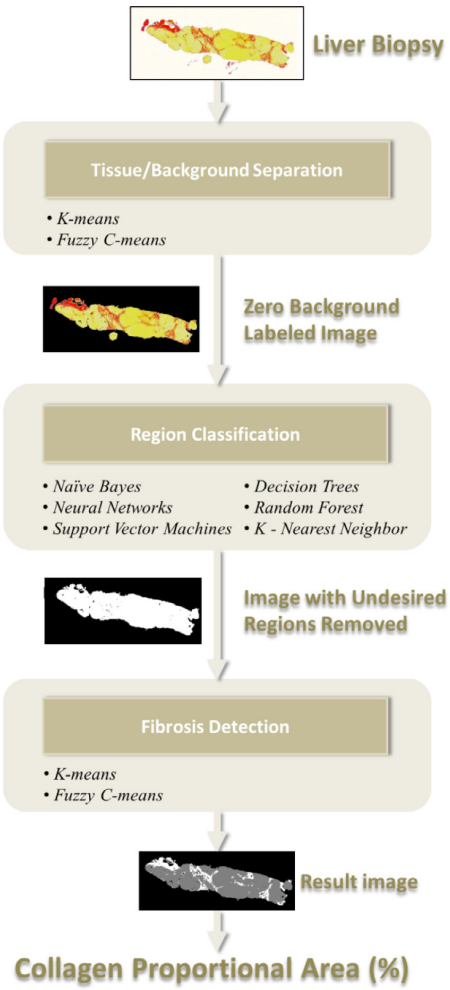

Figure 21: Methodology flowchart[124].

Elaborating further on the methodology; in stage one, the background/tissue separation was performed with the use of a clustering method. With an intention to reduce size and time complexity, each image was divided in 9x9 windows, starting from the top left pixel

of the image. A feature vector with three components was then calculated for each window based on the mean value of each window from RGB channels (intensity based features). Two centroids were then calculated on all feature vectors with the use of a clustering algorithm. The number of clusters was set equal to two, with number one corresponding to background and number two to tissue regions.

KM and FCM clustering algorithms were both tested for stage one. A square-error criterion calculated for each cluster was utilized on the KM algorithm. Every single vector of the image, which is corresponding to a 9x9 window, was assigned to the nearest centroid. Both centroids were then recalculated repeatedly until the distance between the centroids of two subsequent iterations is minimized. Equally, FCM algorithm depends on the minimization of an objective function, though a membership value defines that each feature vector belongs partly in both centroids. After repeated calculations and generation of centroids, a segmented image was produced according to the Euclidean distance, with the assignment of all pixels to the nearest centroid. At the end of the clustering stage there was production of images with tissue regions in zero background. Afterwards, all eight connected regions were identified and labeled, eliminating regions with less than 500 pixels. **(Figure 22)**

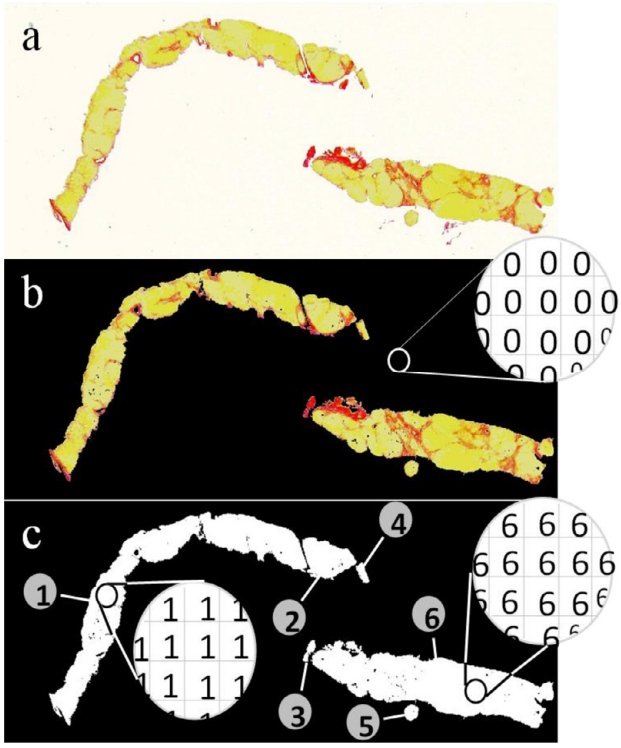

Figure 22: Background/tissue separation stage. a: Liver biopsy image. b: Zero background image. c: Labeled image.[124]

Labeled images included the non-background regions, however, those might have contained numerous artifacts. Therefore, a region classification procedure was performed in order to exclude the artifacts from the CPA assessment. Since the main idea was to separate liver tissue from all the other regions, the image regions were classified as either liver or non-liver tissue. For this classification, a set of features was extracted from each image, including shape and color properties. Shape based features included the following: a. number of pixels in the region (area), b. eccentricity of the ellipse that has the same second-moments as the region (eccentricity), c. diameter of a circle with the same area as the region (diameter), d. number of objects in the region minus the number of holes in the objects (euler number), e. ratio of pixels in the region to pixels in the total bounding box (extent), f. the length (pixels) of the major and minor axis of the ellipse that has the same normalized second central moments as the region (major & minor axis length), g. distance around the boundary of the region (perimeter) and h. proportion of the pixels in the convex hull that are also in the region (solidity). **(Table 6)** The color properties used were the minimum, maximum and average pixel intensity for each RGB channel within the image. Therefore, the calculated feature vector had 18 feature values; 9 shape-based and 9 color-based. Box-plots of the features for liver and non-liver tissue were then created. **(Figure 23)** Thereafter, each feature vector was marked as either liver tissue or undesired region, as per experts' input. This dataset was then used to evaluate and train a classification algorithm. For this second stage, six well-known algorithms were tested in view of identifying the most appropriate: 1) Naïve Bayes classifier (NB). 2) K Nearest Neighbor algorithm (KNN), with K=10 nearest neighbors and the normalized Euclidean distance as distance function. 3) Decision trees (DT), based on the C4.5 algorithm. The pessimistic error rate method with sub-tree replacement was employed for pruning, with at least 2 instances per leaf and 0.25 confidence factor. 4) Random Forests (RF), which was created as an ensample of 10 random trees. 5) Neural Network (NN), which was implemented as a Multilayer Perceptron, with 2 hidden layers, each of them having 10 neurons. 6) Support Vector Machines (SVM), with a polynomial kernel[125,126].

The result was the prediction of the type of tissue for each region. The undesired regions were then removed from the label image and the remaining regions classified as liver tissue were used for the third stage of the methodology.

Shape–based features	Description
Area	Number of pixels in the region
Eccentricity	Eccentricity of the ellipse that has the same second–moments as the region
Diameter	Diameter of a circle with the same area as the region
Euler number	Number of objects in the region minus the number of holes in the objects
Extent	Ratio of pixels in the region to pixels in the total bounding box
Major & minor axis length	The length (pixels) of the major & minor axis of the ellipse that has the same normalized second central moments as the region
Perimeter	Distance around the boundary of the region
Solidity	Proportion of the pixels in the convex hull that are also in the region

Table 6: Region shape-based features.[124]

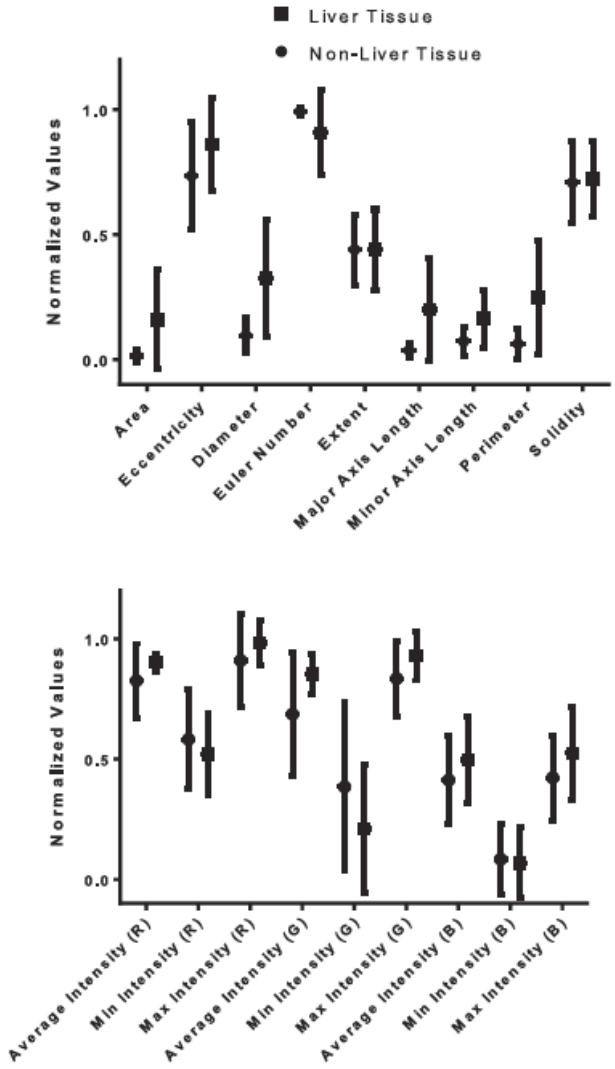

Figure 23: Background/tissue separation stage. a: Liver biopsy image. b: Zero background image. c: Labeled image.[124]

Fibrosis detection was then performed via clustering using either KM or FCM algorithms. The RGB values of each pixel were used, whilst the number of clusters was set, as before, to two; one for normal liver tissue and two for fibrotic tissue. Following the clustering, the segmentation results were merged with the background detection, as produced in the first stage. This resulted in an image, which provided characterization in pixel level, comparably with the experts'-medical annotation. The image had three colors: 1) black for the background pixels, 2) gray for the normal tissue pixels and 3) white for the fibrosis pixels. **(Figure 24)**

Collagen Proportional Area or CPA was then calculated as the ration between the number of fibrosis pixels (from stage three) and the number of pixels from liver tissue regions (from stage two). The mathematical equation used was the following:

$$CPA = \frac{n_{fibrosis}}{n_{fibrosis} + n_{normal\ liver\ tissue}}, (\%) \quad \textbf{(1)}$$

where $n_{fibrosis}$ is the number of fibrosis pixels and $n_{normal\ liver\ tissue}$ is the number of normal liver tissue pixels.

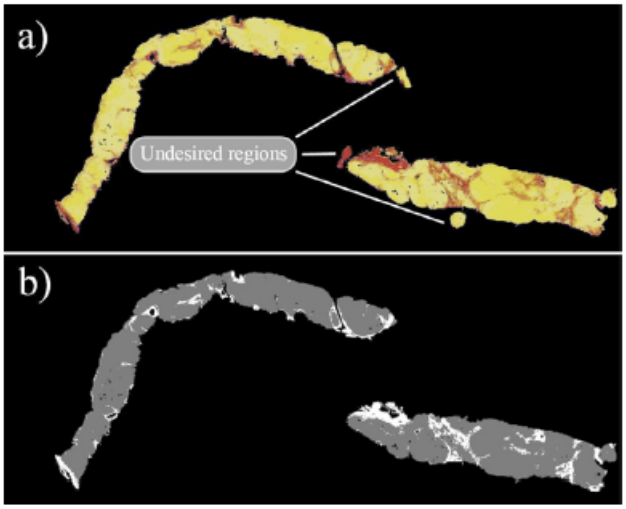

Figure 24: Fibrosis detection stage. a: Zero background image. b: Resulting image (gray areas = normal liver tissue, white = fibrosis expansions)[124]

CHAPTER B3: Statistical Analysis and obtained results

The methodology was tested with the use of the KM and FCM clustering algorithms in stage one and three (no combinations were tested) and with the NB, KNN, DT, RF, NN and SVN classification algorithms in stage two. In the second stage, each algorithm was applied to the dataset with the use of ten-fold stratified cross-validation procedure with regards to the number of regions. The obtained result for each region was then used as the final classification result for this stage and was either rejected, if classified as non-liver tissue, or further processed in stage three, if classified as liver tissue.

The end result of the proposed methodology was the calculation of the CPA for each region. **(Table 7)** All CPAs produced by the methodology were then compared with the manually calculated CPAs which have used the Bland-Altman plots. **(Figure 25)**

Next step was the calculation of the absolute CPA error, which is defined as:

$$absolute\ CPA\ error = \left|CPA - CPA_{methodology}\right| (\%). \quad (2)$$

Absolute CPA error was calculated for all algorithms (clustering and classification), followed by mean absolute CPA error and absolute CPA error standard deviation. **(Table 8)**

The dataset was further divided in two subsets, based on whether the CPA value of each image was ≤ 10% or > 10%, with the mean value and standard deviation of the absolute CPA error for each subset also calculated. That was based on medical experts reports stating that CPA values ≤ 10% can be more pivotal when deciding for medical treatment, in comparison with values > 10%.

The concordance correlation coefficient (CCC)[136] was used in order to assess the concordance between the CPAs automatically calculated by using the proposed methodology and the manually calculated CPAs. CCC is a metric system used to assess the agreement between two variables. This ranges between -1 to 1, with "1" corresponding to perfect agreement, "0" to no agreement and "-1" to perfect disagreement. All calculated CCC values were greater than 0.7. **(Table 9)**

		KM						FCM					
#	CPA	NB	KNN	DT	RF	NN	SVM	NB	KNN	DT	RF	NN	SVM
1	10.37	9.63	9.63	8.29	9.07	8.06	9.08	9.64	9.64	8.10	7.77	9.09	9.09
2	1.42	1.48	1.47	1.47	1.45	1.53	1.44	1.48	1.47	1.44	1.44	1.52	1.47
3	3.59	4.33	4.31	4.29	4.30	4.29	4.31	4.32	4.29	4.29	4.30	4.29	4.27
4	1.40	2.45	1.74	2.77	2.45	2.48	2.82	2.44	1.75	2.51	2.47	2.47	12.85
5	2.39	43.8	2.02	1.98	38.3	2.41	1.82	44.6	2.07	1.27	1.86	2.14	28.02
6	1.76	1.34	1.41	1.34	1.34	1.34	1.34	1.34	1.41	1.33	1.33	1.33	1.34
7	31.73	30.9	28.61	21.9	28.1	28.6	27.97	31.0	28.69	27.5	28.0	28.6	28.03
8	2.96	3.38	3.37	3.37	3.37	3.37	3.38	3.37	3.36	3.36	3.36	3.37	3.37
9	1.35	2.19	2.19	2.19	2.19	2.19	2.19	2.19	2.19	2.19	2.19	2.19	2.19
10	1.77	1.43	1.43	1.43	1.43	1.43	1.43	1.42	1.42	1.42	1.42	1.42	1.42
11	2.37	2.53	2.53	2.53	2.53	2.53	2.53	2.53	2.53	2.53	2.53	2.53	2.53
12	2.27	1.93	1.93	1.93	1.93	1.93	1.93	1.93	1.93	1.93	1.93	1.93	1.93
13	1.92	1.61	1.61	1.61	1.61	1.61	1.61	1.62	1.62	1.62	1.62	1.62	1.62
14	4.26	4.45	4.41	4.41	4.41	4.41	4.41	4.44	4.41	4.43	4.41	4.41	4.41
15	3.08	3.03	3.03	3.03	3.03	3.03	3.03	3.03	3.03	3.03	3.03	3.03	3.03
16	1.50	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26
17	4.62	5.74	5.49	5.49	5.49	5.49	5.49	5.74	5.50	5.50	5.50	5.50	5.50
18	31.52	32.2	33.67	35.0	32.0	32.7	31.45	31.9	33.67	32.3	34.0	33.4	31.70
19	2.45	2.74	2.96	2.96	2.96	2.96	2.96	2.73	2.86	2.86	2.95	2.86	2.95
20	1.52	2.13	2.45	2.45	2.45	2.45	2.45	2.12	2.45	2.45	2.45	2.45	2.45
21	14.86	16.0	16.07	16.0	16.0	16.0	16.09	16.0	16.07	16.0	16.0	16.0	16.07
22	3.80	3.36	5.07	5.07	5.14	5.20	5.07	3.34	5.04	5.04	5.14	5.04	5.04
23	4.09	2.75	3.99	5.60	3.99	2.66	4.14	2.75	3.99	2.91	3.99	2.31	4.14
24	1.65	1.87	1.87	1.87	1.87	1.87	1.87	1.86	1.86	1.86	1.86	1.86	1.86
25	2.86	2.46	2.52	2.66	2.45	2.59	2.42	2.46	2.52	2.59	2.48	2.42	2.52
26	1.42	1.11	1.12	1.12	1.11	1.12	1.12	1.11	1.11	1.11	1.11	1.11	1.11
27	2.97	3.87	3.89	3.87	3.87	3.86	3.85	3.88	3.89	3.86	3.86	3.86	3.86
28	1.83	1.67	1.67	1.67	1.67	1.67	1.67	1.67	1.67	1.67	1.67	1.67	1.67
29	0.74	0.91	0.90	0.90	0.90	0.90	0.90	0.91	0.90	0.90	0.90	0.90	0.90
30	1.79	3.24	3.20	3.20	3.20	3.20	3.20	3.22	3.19	3.19	3.19	3.19	3.19
31	2.64	2.79	2.79	2.79	2.79	2.79	2.79	2.77	2.77	2.77	2.77	2.77	2.77
32	38.59	31.2	29.65	29.6	29.5	29.6	29.59	30.9	29.72	29.9	29.7	29.7	29.72
33	1.43	1.31	1.32	1.32	1.32	1.32	1.32	1.30	1.30	1.30	1.30	1.30	1.30
34	2.07	2.57	2.57	2.57	2.57	2.57	2.57	2.45	2.45	2.45	2.45	2.45	2.45
35	2.60	2.73	2.71	2.71	2.71	2.71	2.71	2.74	2.72	2.72	2.72	2.72	2.72
36	1.33	1.85	1.85	1.85	1.85	1.85	1.85	1.85	1.85	1.85	1.85	1.85	1.85
37	1.51	1.05	1.05	1.05	1.05	1.05	1.05	1.06	1.05	1.05	1.05	1.05	1.05
38	1.07	1.44	1.49	1.49	1.49	1.49	1.49	1.44	1.48	1.48	1.48	1.48	1.48
39	1.68	1.29	1.36	1.36	1.36	1.36	1.36	1.28	1.35	1.27	1.35	1.35	1.35
40	12.17	10.4	10.42	10.4	10.4	10.4	10.42	10.4	10.43	10.4	10.4	10.4	10.43
41	11.19	12.2	12.46	12.4	12.2	12.2	12.28	12.2	12.45	12.4	12.2	12.2	12.26
42	2.95	1.73	1.72	1.73	1.72	1.72	1.72	1.73	1.72	1.72	1.73	1.72	1.72
43	9.05	10.1	9.94	10.0	9.94	10.1	9.94	10.1	9.89	9.92	9.90	9.89	9.92
44	3.24	3.82	4.17	3.85	4.17	4.17	4.17	3.80	4.20	4.20	4.20	4.20	4.20
45	6.48	7.43	7.28	7.28	7.28	7.28	7.28	7.39	7.31	7.37	7.31	7.31	7.31
46	10.18	8.89	8.89	8.89	8.89	8.89	8.89	8.89	8.89	8.89	8.89	8.89	8.89
47	4.31	3.65	3.62	3.62	3.62	3.62	3.62	3.70	3.67	3.67	3.67	3.67	3.67
48	4.20	3.42	3.38	3.41	3.38	3.38	3.38	3.43	3.38	3.38	3.41	3.38	3.38
49	3.79	4.04	4.04	4.04	4.04	4.04	4.04	4.03	4.03	4.03	4.03	4.03	4.03
50	6.92	2.93	2.91	2.93	3.28	2.91	2.94	2.92	2.89	2.92	2.88	2.92	2.92
51	3.95	3.23	3.23	3.23	3.23	3.23	3.23	3.23	3.23	3.23	3.23	3.23	3.23
52	5.63	3.81	4.04	4.04	4.04	4.04	4.04	3.81	4.02	4.02	4.02	4.02	4.02
53	2.04	2.50	3.34	2.71	3.07	2.70	2.96	2.51	3.27	3.45	3.06	2.79	2.92
54	2.86	2.59	2.59	2.59	2.59	2.59	2.59	2.60	2.60	2.60	2.60	2.60	2.60
55	2.19	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
56	3.19	1.96	3.02	2.94	2.96	2.96	3.02	1.95	3.01	2.81	2.95	3.01	3.01
57	3.93	1.68	2.89	2.79	2.85	2.79	2.77	1.68	2.89	2.79	2.79	3.08	2.77
58	1.44	1.60	1.59	1.56	1.55	1.65	1.54	1.59	1.60	1.56	1.55	1.67	1.54
59	1.27	1.47	1.47	1.47	1.47	1.47	1.47	1.47	1.47	1.47	1.47	1.47	1.47
60	3.91	2.80	2.79	2.80	2.80	2.80	2.80	2.79	2.78	2.79	2.78	2.79	2.79
61	13.31	13.1	13.12	13.1	13.1	13.1	13.12	13.1	13.11	13.2	13.1	13.1	13.11
62	20.09	21.3	21.26	17.3	21.3	21.3	21.47	21.3	21.24	17.3	22.2	22.2	21.46
63	3.43	2.96	3.09	3.09	3.09	3.09	3.10	2.95	3.09	3.10	3.11	3.10	3.10
64	7.95	5.53	5.53	5.53	5.53	5.53	5.53	5.51	5.51	5.51	5.51	5.51	5.51
65	5.10	3.12	3.12	3.12	3.12	3.12	3.12	3.11	3.11	3.11	3.11	3.11	3.11
66	3.34	2.70	2.88	2.88	2.88	2.88	2.87	2.70	2.86	2.86	2.86	2.65	2.79
67	9.45	6.85	5.90	5.96	5.96	5.96	6.25	6.83	5.89	5.89	5.89	5.95	5.95
68	16.14	16.5	16.53	16.5	16.5	16.5	16.53	16.5	16.52	16.5	16.5	16.5	16.52
69	3.71	2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72
70	3.47	3.28	3.32	3.28	3.28	3.28	3.32	3.28	3.32	3.28	3.32	3.32	3.32
71	4.77	4.12	4.42	5.22	4.62	4.86	27.19	4.12	4.58	4.64	4.89	4.58	28.12
72	2.47	2.94	2.28	2.94	2.32	2.56	2.27	2.95	2.59	2.65	2.58	2.31	2.29
73	23.72	44.1	42.91	43.4	43.0	40.2	42.94	44.1	43.83	42.8	42.7	40.1	42.97
74	17.05	32.4	33.11	32.9	33.1	33.1	33.11	32.6	33.35	32.8	33.3	33.3	33.35
75	6.09	4.66	4.57	4.57	4.57	4.57	4.57	4.62	4.57	4.57	4.57	4.57	4.57
76	2.18	2.98	2.98	2.98	2.98	2.98	2.98	2.97	2.97	2.97	2.97	2.97	2.97
77	1.63	1.55	1.53	1.54	1.53	1.54	1.53	1.55	1.53	1.54	1.54	1.54	1.53
78	3.95	3.53	3.53	3.53	3.53	3.53	3.53	3.54	3.54	3.54	3.54	3.54	3.54
79	1.14	2.28	2.31	2.31	2.31	2.30	2.31	2.25	2.26	2.26	2.26	2.26	2.26

Table 7: Fibrosis percentage manually calculated (CPA) in comparison with all clustering and classification algorithms (KM – NB, KM - KNN, KM – DT, KM – RF, KM – NN, KM – SVN, FCM – NB, FCM- KNN, FCM – DT, FCM – RF, FCM – NN, FCM – SVN)

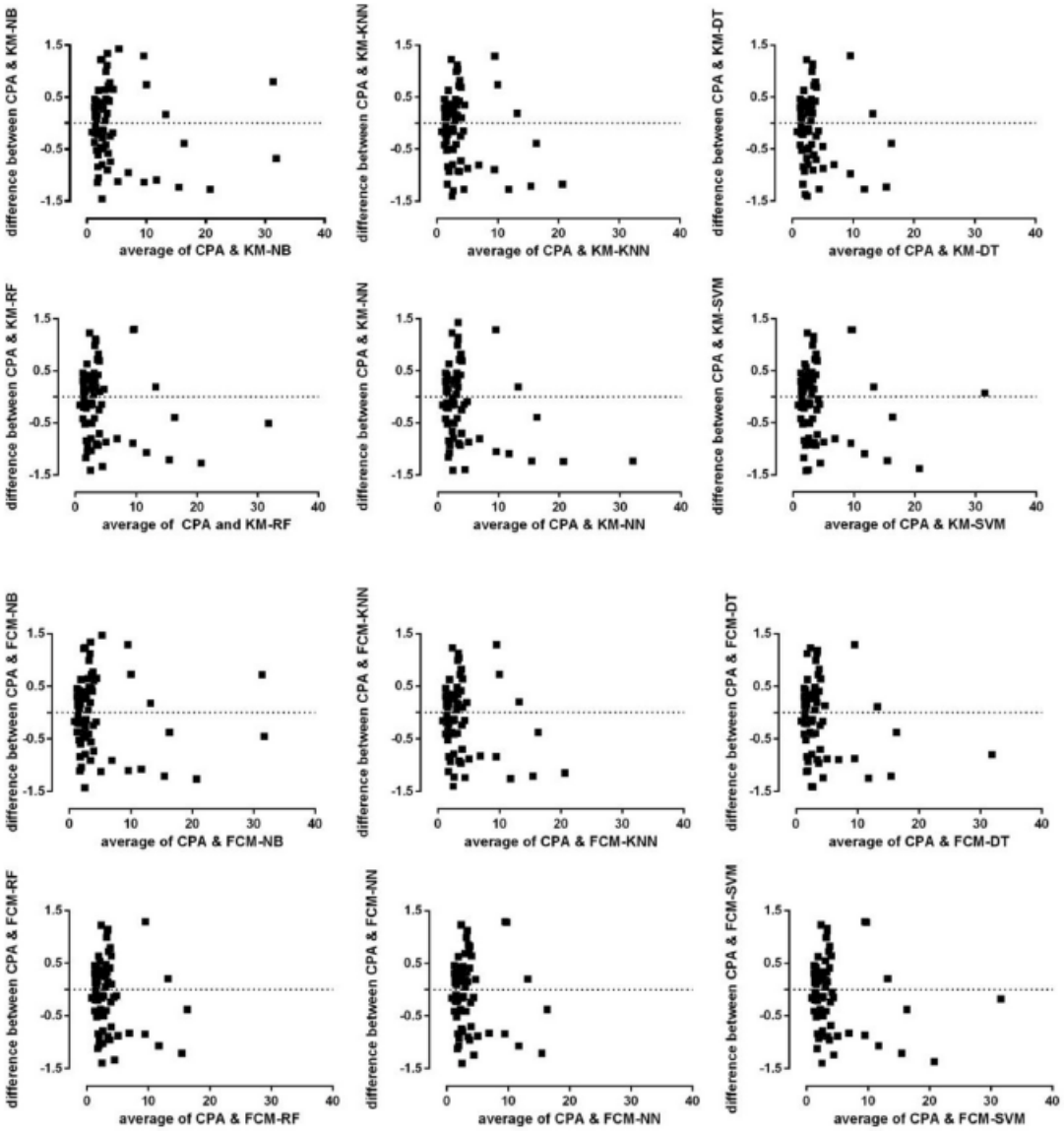

Figure 25: Bland-Altman plots between CPAs calculated from the proposed methodology vs. manually calculated CPAs.[124]

Clustering algorithm	Classification algorithm	Total		CPA ≤ 10%		CPA > 10%	
		Mean	Std	Mean	Std	Mean	Std
KM	NB	1.79	5.36	1.35	5.06	4.04	6.49
	KNN	1.31	2.91	0.69	0.74	4.42	6.30
	DT	1.48	3.10	0.72	0.73	5.30	6.37
	RF	1.75	4.88	1.23	4.40	4.38	6.38
	NN	1.29	2.71	0.71	0.74	4.26	6.36
	SVM	1.58	3.76	1.04	2.77	4.36	5.78
FCM	NB	1.80	5.46	1.36	5.16	4.05	6.36
	KNN	1.31	3.00	0.68	0.74	4.50	6.56
	DT	1.37	2.90	0.73	0.74	4.59	6.52
	RF	1.36	2.92	0.70	0.74	4.69	6.17
	NN	1.31	2.72	0.71	0.74	4.30	6.18
	SVM	2.04	4.82	1.58	4.36	4.37	6.55

Table 8: CPA absolute errors (%) for all different clustering/ classification algorithms[124]

Clustering algorithm	Classification algorithm	Concordance correlation coefficient
KM	NB	0.7735
	KNN	0.9152
	DT	0.8991
	RF	0.7973
	NN	0.9229
	SVM	0.8659
FCM	NB	0.7672
	KNN	0.9116
	DT	0.9125
	RF	0.9138
	NN	0.9231
	SVM	0.7925

Table 9: Concordance correlation coefficient[124]

C. DISCUSSION AND CONCUSIONS

We present a fully automated methodology for calculation of the CPA from liver biopsy images from patients with HCV. This methodology is based on consecutive application of clustering and classification algorithms and was designed to be completely automated and in view of avoiding threshold-based decisions. Moreover, the stages of the methodology are optimized with regards to computational time.

The total CPA absolute error in all different algorithms varied between 1.29% (KM/NN) and 2.04% (FCM/SVM). In the subset of images with CPA ≤ 10%, both mean CPA error and CPA error standard deviation were < 0.75%. **(Table 8)** For patients with CPA≤ 10%, CPA error less than 1 was considered sufficient according to our expert opinion.

Moreover, as already mentioned, the calculated concordance correlation coefficient (CCC) values were high. In particular, with the use of KM clustering algorithm, KM/KNN and KM/NN correlation achieved CCC values higher than 0.9 (0.9152 and 0.9229 respectively), whilst KM/DT combination was close to 0.9 (0.8991). With the use of the FCM algorithm, four out of the six correlation combinations achieved CCC values as high as 0.9231 recorded in FCM/NN combination, which was also the overall best result. **(Figure 26)**

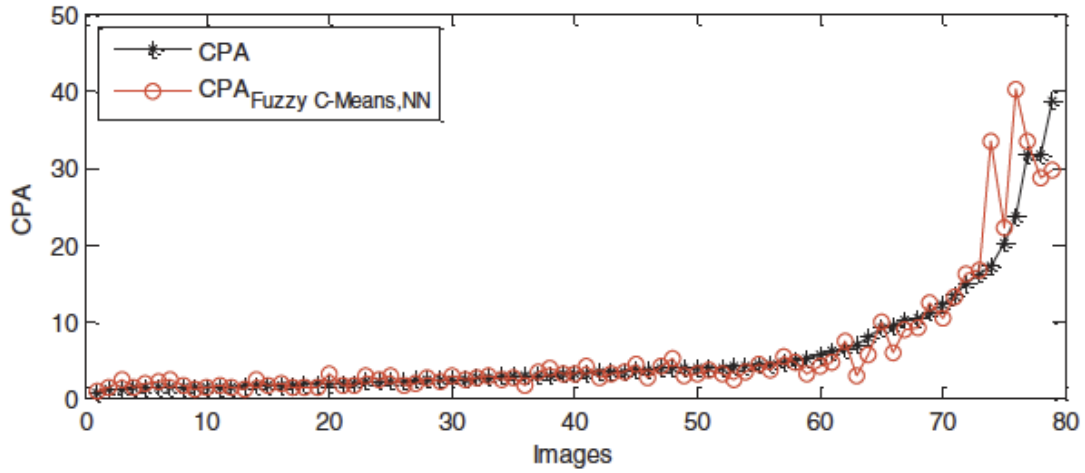

Figure 26: CPA & calculated from the methodology CPA using FCM and NN algorithms for all images, sorted in an increasing CPA value[124]

In total, the obtained results showed less than 0.73% CPA error for CPA≤ 10% in 7 out of the 12 approaches, as well as CCC values >0.89, underpinning the robustness of our proposed methodology.

As already mentioned, the methodology was designed in view of avoiding threshold-based analysis, which involves manual testing and human interaction. In literature, manual threshold setting and manual region selection and removal are more commonly used to separate the tissue/background and identify liver regions respectively. In our methodology a clustering algorithm and feature selection and classification approach was used instead[127–130].

The only threshold used was the 500 pixels limit, which excludes from the study very small images. This step was included in order to accelerate stage 2 as the 500 pixels threshold is used there as well. In stage 2 though, the process is computationally more time consuming and costly. In case those very small images make it to stage 3 of the methodology, their impact in the final CPA estimation is limited given their size. The minimization/elimination of threshold-based image analysis contributes to the development of a methodology exempting subjective decisions. Moreover, in stages 1 and 3 (clustering stages) the liver tissue pixels/ background pixels average ration was 0.201 in stage 1 and the corresponding average ration of fibrosis pixels / liver tissue pixels was 0.058 in stage 3, producing accurate results despite being applied to unbalanced datasets.

This methodology is, as already commented on, designed to optimize processing time. That is due to several reasons: (a) low – resolution images are being processed, where all the tissue from each sample is included in a sole photograph. (b) 9 x 9 areas are used for the generation of the centroids of the liver tissue and background clusters in stage 1, allowing accelerating the centroid calculation procedure. The final clustering is created pixel by pixel. (c) 500 pixels is used as threshold, significantly accelerating stage 2, as it considerably decreases that number of regions that require classification. These mostly correspond to noise, but even in the event that they correspond to liver tissue, they are highly fragmented and should be excluded from the calculation. (d) The main advantage of the methodology is that it is entirely automated.

All other methods used in literature require manual threshold selection or area selection[127,127–129,131]. In the case of area selection, additional time is required from merging multiple images, which necessitates either very sophisticated equipment or further more time.
Additionally, our proposed methodology employs liver biopsy images and analyses them in order to automatically calculate CPA. Liver biopsy images have been clinically validated many times in the past two decades and are considered as a medical index. qFibrosis is a new proposed index though is yet to be validated[131].

In conclusion, we present a fast operating fully automated methodology of image analysis. This can be easily applied in order to obtain low-resolution liver biopsy images in order to extract CPA and can be used in daily clinical practice. The methodology has proven to be very accurate when CPA is <10%, as shown by the mean absolute CPA error, absolute CPA error and standard deviation. However, in cases with CPA >10%, the results indicated lower accuracy. The FCM/NN combination was 4.30% with a 6.18% absolute CPA error standard deviation. This was largely due to the large fibrotic areas within the liver tissue, which are normally manually removed before calculating the CPA.

This study aimed on looking into the automatic calculation of liver fibrosis in patients with HBV as well. Regarding these patients, a similar digital image analysis (DIA) (adjusted for handling liver biopsy images from patients with hepatitis B) was applied. More specifically, in this study we tried to identify fibrosis cutoff in HBeAg negative patients with moderate fibrosis in requiring treatment, as this has not been defined by any staging system. We used 84 biopsies from treatment naïve patients with HBV DNA<20,000IU/mL and ALT<2 ULN. Those were retrospectively evaluated using DIA expressed as CPA and the Ishak system. For the CPA, binary logistics were used adjusted for age/gender, alcohol use, body mass index, diabetes mellitus (DM), AST, ALT, ALP, γGT, bilirubin, albumin, viral load and platelet count. Sensitivity and specificity of the CPA were assessed with the correlation coefficient and the ROC curve. Our cohort of patients had median age of 49 years, 56 were males, 29 reported alcohol use, 19 had diabetes mellitus, their median BMI was 23, median viral load 13.500 IU/ml and median ALT of 54IU/L. The median stage was 2.5 (1-5) and the CPA 5.67% (2-12.6). Age, alcohol, γGT and DM were as univariates associated with stage ≥3. Only γGT could multivarietly predict stage ≥3, with a p value of

0.045, exp(B) 1.03, 95% CI (0.996-1.058). The median CPA for stage 1 was 3.2% (2-5.6) calculated in a total of 22 patients, for stage 2: 3.9% (2.2-9.5) in 25 patients, stage 3: 5.76% (2.7-11) in 17 patients, stage 4: 9.1% (5.9-12) in 13 patients and for stage 5: 10.1% (7.7-12.6) in 7 patients. For the correlation with stage ≥3 AUROC was 0.85 (95% CI 0.76-0.94). Spearman's Rho was 0.73 (p=0.001). CPA 5% was linked with stage ≥3 with sensitivity of 80% and specificity 84%. Based on this CPA cutoff 14% (3 of 22) of patients with Ishak stage 1 and 28% (7 of 25) with Ishak stage 2 had significant fibrosis but had not received treatment as per guidelines. For stage 1 and 2 patients, 1 out of 3 and 4 out of 7 respectively were initiated on treatment within 2 years after the liver biopsy due to an increase in viral load (>20,000 IU/ml).

Based on the above, we concluded that there was good correlation found between CPA measurements and Ishak's stage in patients with chronic hepatitis B. Nevertheless, CPA captured stage 1 and 2 patients with similar collagen content to stage 3. Thus, further evaluation of CPA is required to identify treatment thresholds in patients with chronic hepatitis B using the same DIA technique as in patients with HCV. Also, the same technique could be used and in many other chronic liver diseases as well as in skin diseases etc.

D. ABSTRACT

Extraction of collagen proportionate area (CPA) in liver biopsy images provides the amount of fibrosis in liver tissue. This is the most distinguishing histological feature in viral hepatitis. Staging is currently based on semi-quantitative scores, such as the Ishak and METAVIR. CPA calculation based upon image analysis techniques has since proven to be more accurate than semi-quantitative scores. Though, lack of standardization and robust methodologies for assessment of computerized image analysis for CPA has proven to be a major limitation and hence CPA has not yet reached daily clinical practice.

This current work proposes a fully automated methodology for CPA extraction. It is based on machine learning techniques and is composed by three stages. In particular, background tissue separation and fibrosis detection in regions of liver tissue has been performed with the use of clustering algorithms. Classification algorithms have also been employed in order to differentiate between liver tissue regions and non-liver tissue regions, such as structural collagen, muscle tissue and blot clots. The non-liver tissue regions have been then excluded from the CPA computation.

The methodology was evaluated with the use of 79 liver biopsy images from patients with hepatitis C. The obtained mean absolute CPA error was 1.31% with a concordance correlation coefficient of 0.923.

Manual threshold-based and region selection processes, which are widely used in literature, are being avoided with the proposed methodology. Moreover, the CPA calculation time is minimized.

In regards to the use of the technique in patients with hepatitis B, we concluded that digital image analysis requires further evaluation.

Ε. ΠΕΡΙΛΗΨΗ (ΕΚΤΕΤΑΜΕΝΗ ΠΕΡΙΛΗΨΗ) ΣΤΗΝ ΕΛΛΗΝΙΚΗ ΓΛΩΣΣΑ

Η ποσοτικοποίηση του κολλαγόνου ως αναλογία κολλαγόνου στη βιοψία ήπατος (Collagen Proportionate Area – CPA) σε βιοψίες ήπατος παρέχει το βαθμό της ηπατικής ίνωσης. Αυτό αποτελεί το πιο τυπικό ιστολογικό χαρακτηριστικό στις ιογενείς ηπατίτιδες. Η σταδιοποίηση επί του παρόντος βασίζεται σε ημιποσοτικά συστήματα ταξινόμησης, όπως το Ishak και το METAVIR. Ο υπολογισμός της CPA με τεχνικές ανάλυσης εικόνων αποδείχτηκε πιο ακριβής σε σύγκριση με τα ημιποσοτικά συστήματα ταξινόμησης. Ωστόσο, η έλλειψη τυποποίησης και ισχυρών μεθοδολογιών για την αξιολόγιση της ψηφιακής ανάλυσης εικόνων για CPA αποτελεί σημαντικό περιοριστικό παράγοντα και ως εκ τούτου η CPA δεν χρησιμοποιείται ακόμα στην καθημερινή κλινική πρακτική.

Η παρούσα εργασία προτείνει μια πλήρως αυτοματοποιημένη μεθοδολογία για την εξαγωγή της CPA, η οποία βασίζεται σε τεχνικές μηχανικής μάθησης (machine learning) και περιλαμβάνει τρία στάδια. Πιο συγκεκριμένα, ο διαχωρισμός του φόντου από τους ιστούς όπως επίσης και η ανίχνευση ίνωσης σε περιοχές (regions) ηπατικού ιστού πραγματοποιήθηκε με τη χρήση αλγόριθμων ομαδοποίησης (clustering algorithms). Για το διαχωρισμό περιοχών ηπατικού από μη ηπατικούς ιστούς, όπως για παράδειγμα δομικό κολλαγόνο, μυικό ιστό και θρόμβους αίματος, χρησιμοποιήθηκαν αλγόριθμοι κατηγοριοποίησης (classification algorithms). Στη συνέχεια, οι περιοχές μη ηπατικού ιστού αποκλείστηκαν από τον υπολογισμό της CPA.

Η μεθοδολογία αξιολογήθηκε με τη χρήση 79 εικόνων από ηπατικούς ιστούς ασθενών με ηπατίτιδα C. Το μέσο απόλυτο σφάλμα (mean absolute error) υπολογίστηκε σε 1.31% ενώ ο συντελεστής συμφωνίας συσχέτισης (concordance correlation coefficient) σε 0.923.

Στην υπάρχουσα βιβλιογραφία η επιλογή περιοχών αλλά και η εισαγωγή ορίων πραγματοποιείται με χειροκίνητες διαδικασίες. Οι διαδικασίες αυτές αποφεύγονται με την προτεινόμενη μεθοδολογία. Επιπλέον, ο χρόνος υπολογισμού του CPA περιορίζεται στον ελάχιστο δυνατό.

Όσον αφορά τη χρήση της τεχνικής σε ασθενείς με ηπατίτιδα B, καταλήξαμε στο ότι απαιτείται περαιτέρω εκτίμηση.

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