NON INVASIVE EVALUATION
OF
LIVER FIBROSIS
IN
PATIENTS WITH CHRONIC
HEPATITIS B AND C

(Ph.D. Thesis)

SHAHZAD ASHRAF
MBBS
Diplomate American Board of Internal Medicine

Dept of Medicine
Baqai Medical University
2006
Supervisor

Professor Lt Gen (R) Syed Azhar Ahmed
Hilal-e-Imtiaz (M), Sitara-e-Basalat
MBBS, Ph.D. (London), FRCPath (London), FCPS
Vice Chancellor
Baqai Medical University

Co Supervisor
Professor Jameel Ahmed
MBBS, MRCP
Professor of Medicine
Baqai Medical University
This work is dedicated to

My parents & wife
for their constant encouragement and support

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ABSTRACT
ABSTRACT

Chronic viral hepatitis is an important cause of morbidity and mortality in the present day world [Goldstein et al, 2005; Baldo et al, 2008]¹,². The situation is particularly precarious in the developing countries [Jafri et al, 2006]³. It is estimated that by the year 2020-5, there will be three fold rise in cirrhosis, and hepatocellular carcinoma from HBV and HCV [Nguyen et al, 2008; Law et al, 2003]⁴,⁵. In chronic viral hepatitis the prognosis and management are highly dependent on the extent of liver fibrosis [Sebastiani et al, 2006]⁶. Though classically considered the “gold standard”; the liver biopsy is far from perfect, and has significant limitations [Poynard et al, 2004]⁷. This has led researchers to look for other methods to assess the stage of liver fibrosis [Afdhal et al, 2004]⁸. The noninvasive markers are the most widely used alternative to liver biopsy [Manning et al, 2008; Castera et al, 2007; Morra et al, 2007]⁹,¹⁰,¹¹.

In the study presented, the association between serum markers, platelet parameters and liver fibrosis was investigated taking liver biopsy as the reference standard. A set of 5 serum markers, Fibroscore, consisting of: bilirubin, gamma glutamyl transferase (GGT), hyaluronic acid (HA), alpha 2 macroglobulin (A2M), and platelets; has shown very high diagnostic accuracy for the near absence of fibrosis, and cirrhosis. The area under the ROC for F2 (stage 2) fibrosis was 0.808, for F3 the ROC was 0.938, and for F4 the ROC was 0.959. A central cut off point of > 0.5, in the model, predicted clinically significant fibrosis, (F2, F3 and F4) with a sensitivity of 82%, specificity of 92%, and overall diagnostic accuracy of 89%. By increasing the cut off to 0.65, for stages F2-F4, the PPV was 95%. Lowering the cut off to < 0.08 for the exclusion of stages F2-F4 provided 98% NPV, thus almost certainly ruling out stages F2-F4. The PDW index consists of platelet distribution width (PDW), mean platelet volume (MPV), and platelet count. The area under the ROC for advanced fibrosis (F3-F4) for PDW index was 0.840, compares with the well known AST to Platelet Ratio Index (APRI) with area under ROC of 0.888.

It is concluded that, Fibroscore has a high diagnostic accuracy for stages F2-F4, and PDW Index reliably predicts advanced fibrosis. The noninvasive markers will be helpful in the screening and management of fibrotic liver disease [Morra et al, 2007]¹¹, and will replace liver biopsy in most patients with chronic liver disease from viral related causes [Castera et al, 2007; Morra et al, 2007]¹⁰,¹¹.

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1. Introduction

Chronic liver diseases, CLD, are a major cause of morbidity and mortality in the present day world 1-6. Chronic infections with hepatitis B and C viruses, alcoholic and non-alcoholic fatty liver diseases are important causes of CLD 3,7-13. The major determinant in their prognosis is the progressive accumulation of fibrosis, with distortion of the hepatic architecture, and ultimate progression to cirrhosis and its allied complications 3,8,9,14-21. In the recent years, with the great advancement in therapeutic modalities to treat chronic liver diseases, accurate assessment of fibrosis has become of paramount importance; to guide management decisions, predict outcome (prognosis), and monitor disease activity in individual patients 21-26.

1.1 Burden of chronic hepatitis

HBV is the most common cause of cirrhosis and hepatocellular carcinoma in the world today. Of approximately 2 billion people who have been infected with HBV worldwide, more than 350 million, or about 5% of the world’s population are chronic carriers, and with an annual incidence of more than 50 million 1,27. HBV accounts for 500,000 to 1.2 million deaths per year 4.

Similarly more than 3% of the world population, or about 170-5 million people may be infected with HCV 2,3,28,29. The prevalence of HCV is increasing and estimates of the future burden of chronic hepatitis C predict at least a 3 fold rise in chronic liver disease and cirrhosis by the year 2020 3,30-32.

Nonalcoholic fatty liver disease (NAFLD) affects 10-30% of the general population in various countries. 10% of these people, or ~ 2-3% of the general population, satisfy the criteria for nonalcoholic steatohepatitis, (NASH). The prevalence of NAFLD is also expected to rise in developed countries given the epidemic of its major determinant, obesity 6,11,12,20,25,33-36.

Studies indicate that advanced fibrosis and cirrhosis will develop in 20-40% of patients with chronic hepatitis B or C and in a similar proportion of patients with nonalcoholic fatty liver disease 3,4,5,7,9,10,11,12,37,38.
1.2 Liver fibrosis

Fibrogenesis is a ubiquitous process in chronic inflammatory diseases in human beings, in response to injury. Progressive fibrosis with the development of cirrhosis, is a complication of all chronic liver diseases whatever their etiology, whether viral, autoimmune, biliary, toxic, or metabolic disease 13,17,18,19,24,39.

Fibrogenesis is a non-specific mechanism, which lasts as long as injury persists and is believed to help limit the extension of inflammatory reaction. Fibrosis, therefore, is a physiologic mechanism, which is at first beneficial, but becomes pathological if viral infection and chronic hepatocellular injury persist 13,16,40.

Fibrosis is the deposition of extracellular matrix, ECM, components within the liver parenchyma. It is the consequence of a dynamic mechanism of gene transcription and protein synthesis of ECM compounds termed fibrogenesis 17,18,40,41.

In recent years, substantial progress has been made in our understanding of the pathophysiology of extracellular matrix, ECM, deposition and metabolism. Extracellular matrix, is a complex mixture of glycoproteins (collagen, elastin, fibronectin, laminin) and proteoglycans organized into complex polymers, which are insoluble and arranged in a tridimensional network that induces loss of liver architecture 13,16,40-44. Basement membranes are usually absent along the sinusoid but are deposited during the process of cirrhosis, a process termed ‘sinusoid capillarisation’ 45.

The collagens are the most important molecular targets, since 1) they represent the major matrix proteins, 2) they form important mechanical scaffolds, and 3) their proteolysis by specific proteases appears to be the rate limiting for ECM removal. In cirrhosis the collagen content increases 10 fold. In normal liver, ECM comprises less than 3% of area on a liver cut section. In cirrhosis a quantitative increase of up to 30%-40% and composition modification is observed. Other major compounds of liver ECM are glycoproteins such as laminin and fibronectin, elastic fibers, and proteoglycans 13,18,42,44,46.

It is now clear that ECM metabolism is a very dynamic process and that deposition of ECM is much more reversible than was previously thought. Hepatic stellate cells are the major source of extracellular matrix deposition, although other cell types such as portal fibroblasts may play a role 19,24,40,47,48. Hepatic stellate cells are located
within the perisinusoidal space (or space of Disse) between the endothelial wall of the sinusoid and the vascular face of the hepatocytes.\textsuperscript{49}

The mechanism of hepatic stellate cell activation has been divided into two steps: initiation and perpetuation, both are closely integrated.\textsuperscript{50} Factors that trigger the initial step of the process are initiators. After HSC’s activation is initiated, they engage in the process and gain an activated phenotype, this phenotype is maintained because of the perpetuation mechanism involving other growth factors.\textsuperscript{13,51}

The HSC’s can be activated by several cytokines (e.g. tumor growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), platelet derived growth factor (PDGF), which are secreted in response to liver injury. On the other hand, other signals, e.g. interleukin-10 (IL-10) promote ECM degradation. Once activated, HSC’s secrete cytokines such as metalloproteinases, TGF-β1, PDGF, monocyte chemotactic protein 1 (MCP-1), and endothelin (ET-1). Some of these are directly involved in the fibrogenesis (TGF-β 1, connective tissue growth factor), others in chemotaxis (MCP-1) and proliferation of HSC’s (PDGF, ET-1), and others in matrix degradation (metalloproteinases).\textsuperscript{13,19,24,40,41}

A variety of adverse stimuli such as toxins, viruses, bile stasis, and hypoxia can trigger fibrogenesis,\textsuperscript{18} and its pathogenesis is somehow related to the etiology of the underlying CLD.\textsuperscript{24}

The mechanisms by which HBV and HCV induce liver fibrosis are only partially understood. In chronic hepatitis B infection the pathogenesis of hepatic fibrosis has been associated with cytokines, particularly TGF-β.\textsuperscript{152} HCV on the other hand induces oxidative stress and recruitment of inflammatory cells, with HSC’s activation and collagen deposition. In addition several HCV proteins directly stimulate the fibrogenic pathways of HSC’s.\textsuperscript{53} By interaction with mitochondria, core protein induces reactive oxygen intermediates and thus oxidative stress which can induce steatohepatitis.\textsuperscript{54,55} Hepatitis C virus nonstructural genes (NS3 and NS5B) are able to induce increased expression of TFG-β1 and of other profibrogenic factors in infected hepatocytes.\textsuperscript{56} These cellular events directly mediated by HCV in infected hepatocytes could explain the occurrence of progressive liver fibrosis with minimal inflammation.\textsuperscript{24}
In all forms of chronic liver disease, including chronic hepatitis C, the development of fibrosis is a step by step process starting from minimal fibrosis limited to in and around the portal tracts (periportal or zone 1 fibrosis), followed by more extensive fibrosis extending in to the lobules in the liver parenchyma towards the central veins (zone 3), which can form bridges between two portal tracts or portal tracts and central veins eventually ending in cirrhosis\textsuperscript{57,58,59}.

In some patients, the rate of fibrosis is rapid so that cirrhosis eventually develops, and, with it the major complications of chronic viral hepatitis; end stage liver disease, portal hypertension, and hepatocellular carcinoma. In other patients fibrosis does not develop or progresses so slowly that after decades of infection, little or no fibrosis is found on liver biopsy. Such patients are unlikely to suffer the long term complications of chronic viral hepatitis\textsuperscript{16}.

For e.g., in a retrospective study for the natural evolution of chronic hepatitis C; 1/3\textsuperscript{rd} of the patients progressed to cirrhosis in approximately 20 years, 1/3\textsuperscript{rd} within approximately 50 years, and 1/3\textsuperscript{rd} didn’t show any sign of progression\textsuperscript{60}.

In a mathematical model developed in a large number of patients with single liver biopsy; the suggested average (median) rate of progression of fibrosis in chronic hepatitis C was 0.13 Metavir points per year\textsuperscript{61}. Based on this rate, the average patient would develop cirrhosis in 30 years. The rate of progression was higher in men, age > 40 years, and heavy alcohol drinkers >50g/day. Moreover, the time required to progress from stage 1 to 2 may be far longer than the time required to progress from stage 3 to 4 ( or vice versa)\textsuperscript{16}.

For these reasons, clinicians and patients require accurate information about the degree of fibrosis to guide management decisions, monitor disease activity, and make an assessment of prognosis\textsuperscript{23}.

1.3 Anti fibrotic treatment

The best way not to have fibrosis, is not to have CLD altogether; the old dictum, ‘prevention is better than cure.’ The next best option is the treatment of the underlying cause of CLD, for e.g., chronic HBV and HCV. While immunosuppressive cytokines are usually profibrogenic, the antiviral interferons are antifibrogenic. Interferon-\( \alpha \) blocks
activation, proliferation and collagen synthesis of hepatic stellate cells and myofibroblastic cells. Retrospective analyses and also prospective studies in patients with chronic hepatitis C suggest that Interferon-α therapy can prevent fibrosis progression, even in nonresponders to antiviral therapy.

Recently, new targets for therapies aimed at preventing or inhibiting liver fibrosis independent of the underlying disease aetiology are being explored. Animal studies have identified a number of potentially important therapeutic targets, which include glucose regulation, leptin, endothelin, angiotensin II, and a possible role for PPARγ (peroxisome proliferator-activated receptors). All of these can be modulated using a variety of currently available therapies.

New understanding and insights into the pathogenesis of hepatocellular damage, hepatic fibrogenesis, and the development of molecular techniques; such as new viral or non-viral vector systems, RNAi, ribozyme and antisense technologies have made it possible to modulate the expression of specific genes involved in the key pathways of liver injury and fibrosis. For instance by blocking the Fas-ligand activity by siRNA or antisense against Fas receptor almost completely prevented the Fas ligand-induced fulminant liver failure and animal mortality. It has been an important and noteworthy observation. It is also striking that silencing of a key enzyme, caspase 3 or 7, in an apoptotic cascade by siRNA blocks the cell damage.

Alternatively, when the overexpression of fibrogenic cytokines, such as TGF-β and PDGF and their receptors are inhibited at the mRNA level with antisense, ribozymes or siRNA, or interrupted by specific antibodies or protein molecules; the interventions result in marked amelioration of hepatic fibrosis. Moreover, delivery of collagenases genes by adenoviral vectors promotes the resolution of excessive deposition of ECM components and reverses hepatic fibrosis.

The transition of these promising molecular therapies from animal models to clinical trials for the validation of their clinical efficacy and adverse effects in the target patient population usually will take a long period of time.

Human studies are urgently needed to evaluate the effectiveness of therapies aimed at each of the molecular and genetic targets. Furthermore, it is vital that noninvasive methods for the assessment of liver fibrosis be developed and validated, so
that the impact of any new therapies can be readily assessed in a simple way and subsequently followed up easily\textsuperscript{19}.

### 1.4 Evaluation of liver fibrosis

According to Afdhal NH \textsuperscript{22}, one of the major problems faced by the hepatologists and gastroenterologists today is: how best to evaluate and manage the increasing burden of patients with chronic viral and likely non-alcoholic fatty liver hepatitis.

In the last decade, advances in serologic and virologic testing and improvements in therapy have led more patients to be identified and to seek treatment. Unfortunately, despite our improved understanding of the mechanisms involved in ECM deposition, the complex nature of the process continues to be elusive. Little progress has been made in improving either our ability to determine the degree of hepatic injury, particularly fibrosis, or to predict the risk of disease progression for the individual patient \textsuperscript{22}.

Understanding the pathogenesis of hepatic fibrosis on a molecular level has led to the identification of several putative serum markers of hepatic fibrosis. Either individually or in combination, these serum markers appear capable of determining early and advanced hepatic fibrosis. It is vital that new noninvasive methods for the assessment of liver fibrosis be developed and validated, so that the impact of any new therapies can be rapidly assessed in a simple and meaningful way. This information is still provided by the old fashioned liver biopsy. The clinicians rely on the liver biopsy for both prognostic and therapeutic decision making, which can have a major impact on patient’s life \textsuperscript{22}.

A review of the accuracy of liver biopsy in the assessment of liver fibrosis is necessary, as this has been used as the ‘gold standard’ for almost all studies of noninvasive markers of liver fibrosis \textsuperscript{19,22}; but this ‘gold’ appears to have tarnished, in certain respects.
1.5 Liver biopsy

There is no ideal reference for the assessment of liver histology. The diagnosis of liver fibrosis has traditionally relied on liver biopsy. But for both the physician and the patient, the decision to proceed with liver biopsy is not a trivial one. Patients are reluctant to submit to repeated biopsies, which limits our ability to monitor disease progression and effects of treatment. Recent studies have suggested that there can be up to a 33% error in the diagnosis of cirrhosis. Liver biopsy, which is considered the ultimate standard, has three major limitations: 1) a risk of adverse events, 2) sampling error, and 3) intra and inter observer variability.

Over view of the current literature summarizes the risk of liver biopsy as pain (~ 30%), severe adverse events (3 in 1000), prolonged hospital stay 1-5%, and death (3 in 10,000). Most, but not all, studies have shown that the incidence of complications is related to both the prevalence of relative contraindications as well as the number of biopsies taken.

Sampling variability is the major source of variation. Chronic hepatitis does not affect the liver uniformly. The extent of fibrosis may vary from one part of the liver to another; this is especially true of macronodular cirrhosis. The fibrosis starts in and around the portal tracts, and then extends into the liver parenchyma towards the central vein. Liver biopsy samples only 1/50,000th of the mass of liver, and carries a substantial sampling error.

Both autopsy and laparoscopic studies have evaluated the accuracy of liver biopsy for staging fibrosis and diagnosing cirrhosis. These studies have clearly shown that cirrhosis can be missed on liver biopsy in 10-30% of the cases. The majority of this error is due to the understaging of the disease and is more common in macronodular cirrhosis.

Cirrhosis is macronodular if nearly all of the nodules size is greater than 3 mm, and micronodular if nearly all of the nodules size is less than 3 mm. Nodularity and septa may be readily evident in specimens from micronodular cirrhosis, whereas more subtle criteria have to be relied on in macronodular cirrhosis.

Since the diameter of the biopsy needle is less than 3 mm – the size of a nodule, there is always a chance of missing mild to moderate fibrosis. For instance, the biopsy sample may be obtained along the line of portal-portal or portal-central fibrosis; or may
be tangential to it. Thus in the latter case, apart from the extensive fibrosis, other forms of portal/periportal, or even bridging fibrosis between portal tracts or central veins may be under represented. Thus it is recommended that the most adequate biopsy sample should have 11 complete portal tracts, apart from the adequate length.

In addition to the underlying disease severity, the ease with which cirrhosis is diagnosed further depends on the type of specimen (surgical vs needle), type of needle (Menghini or Trucut), approach (intercostal or transjugular), and the quality of applied reticulin stains. A further complicating factor is that biopsy is just a snap shot in time of the dynamic nature of the process, and progression and regression cannot be determined by a single biopsy.

Both the size of the biopsy sample and number of biopsies taken has a major impact on accuracy. Earlier, it was suggested that an adequate biopsy should be at least 15mm in length and contain greater than 5 portal tracts. Recent studies have concluded that an adequate specimen should be at least 20 mm in length with at least 11 complete portal tracts; while others are recommending biopsy samples up to 25 mm in length, and it is suggested that; the bigger is better. The need for obtaining a larger liver biopsy sample of adequate size, contrasts with the patients’ requirement of a procedure causing limited pain and hemorrhagic risks.

It has been reported that the correct diagnosis of cirrhosis increased from 80% with a single biopsy to 100% when three specimens were analyzed. To the contrary, it has also been reported that when biopsies are taken from more than one site the rate of discordance between biopsies may be substantial. For instance, biopsies taken at laparoscopy from both the right and left lobes were compared. It was reported that there was a difference in the Scheuer stage of at least one grade in 33% of the patients. Furthermore, in 14.5% of cases, the diagnosis of cirrhosis was made in one lobe but not in the other.

Similarly intra- and inter-observer variability can lead to over- or under-staging. Even when an experienced physician performs the liver biopsy, and an expert pathologist reads and interprets the findings, up to a 20% error rate in disease staging has been reported. It has also been observed that the experience of the pathologist, as indicated by the longer duration of practice or belonging to an academic setting, may
have an outstanding impact on the diagnostic interpretation of liver biopsy, even higher than determined by the sample size\textsuperscript{93}. The type of needle used to perform the biopsy is also important. Data indicates that cutting type needle (Trucut) is superior to other type (Menghini) in obtaining a better representation of liver fibrosis particularly those in the advanced disease\textsuperscript{94, 95}. Similarly, as expected, a thick needle is superior to the fine needle in assessing the presence of advanced fibrosis and cirrhosis\textsuperscript{96}. The criteria used to evaluate liver biopsy are equally important. The old classification of chronic hepatitis made a rough grading distinction between milder and more severe forms of liver disease. More recently, new insights in the aetiology and therapy of CLD’s, particularly viral hepatitis, has led to a revised classification, aimed to describe and quantify in more details the necroinflammation and fibrosis. Several semiquantitative scoring systems have been proposed to measure the activity grade of inflammation and to stage the amount and type of fibrosis in the liver\textsuperscript{24}. The use of more standardized grading systems of hepatic fibrosis including the Knodell\textsuperscript{97}, later modified by Ishak\textsuperscript{98}, METAVIR\textsuperscript{77}, Scheuer\textsuperscript{99}, and the Batts and Ludwig\textsuperscript{100} scores amongst others have improved the reproducibility of these criteria. Many studies have shown good to excellent inter- and intra-observer reproducibility for the staging of fibrosis, but that the reproducibility of inflammatory scores is significantly worse\textsuperscript{101-105}. Although the METAVIR scoring system for liver fibrosis, has been frequently used in recent times particularly for chronic hepatitis C\textsuperscript{24}, lack of a uniform scoring system of fibrosis adds to the observer bias and difficulties in comparison between studies. Whichever score is used, the histological staging of liver fibrosis on biopsy is artificially represented as a quantitative categorical variable with a linear quantum progression in severity from 0 to 4 or 6. This does not accurately reflect the dynamic biological process of fibrosis. Fibrosis progression is likely to be nonlinear and there is not equal temporal progression between sequential stages\textsuperscript{23}. So all the scoring systems have some limits: being semiquantitative, not linear, prone to intra- and inter-observer variation, to sampling variability, and numbers with linear progression not reflecting the active and dynamic process of fibrosis\textsuperscript{23,106}. 
Although the cost of liver biopsy is not a major factor in our country; yet it can be elsewhere. A cost benefit analysis showed that in US the cost of a liver biopsy is $1032 and it could rise to $2745 when complications occur\textsuperscript{107}. So the procedure is invasive, costly, and difficult to standardize\textsuperscript{24}.

As we certainly are daunted at the prospect of performing 3 million liver biopsies with the associated cost, manpower issues, and risks for the patient, we need to have a reliable alternative, which, if not better can just as effectively guide our decision\textsuperscript{22}.

### 1.6 Noninvasive markers of liver fibrosis

In the recent years, there has been an increasing interest in identifying and describing liver fibrosis through noninvasive surrogate markers measurable in the peripheral blood. Serum markers of liver fibrosis offer an attractive alternative. They are less invasive than biopsy, with no risk of complications, eliminate sampling and observer variability which is in the case of liver biopsy (but there is inter-laboratory variability in the measurement of noninvasive markers), may allow dynamic calibration of fibrosis, can be performed repeatedly, and may be more cost effective in certain parts of the world\textsuperscript{23}.

Most noninvasive markers of liver fibrosis were developed with the aim of discriminating between ‘insignificant’, (F0-F1) by METAVIR and clinically “significant” fibrosis (≥ F2) by METAVIR or of identifying or excluding established cirrhosis in patients with well compensated CLD. Both these aims are clinically the most relevant.

The major clinical utility of the index biopsy in HCV is to enable the clinician to determine the need for treatment. Because of the complexity and side effects of interferon-based therapies, the liver biopsy has taken an increasing role in the clinician’s decision whether to treat a patient. Presence of significant fibrosis in the liver is indeed considered as the hallmark of a progressive liver disease and a clear indication for immediate initiation of antiviral therapy, in agreement with International Guidelines and recommendations for the management of these conditions\textsuperscript{22,108,109}. On the other hand, patients with F0-F1 fibrosis usually do not progress or progress much slowly\textsuperscript{110,111} and are often not as aggressively offered treatment\textsuperscript{22}. 
However as therapy for HCV improves, the clinical need for a biopsy may be less apparent. For example, genotype 2 and 3 patients have a greater than 70% sustained virologic response with pegylated interferons and ribavirin, and in uncomplicated cases a rationale can be made to treat all patients without liver biopsy and only to biopsy those who fail treatment. As newer, better tolerated, and more efficacious therapies are being developed, the need for biopsying all HCV patients to grade and stage the disease may become redundant. Therefore the development of noninvasive tests that can differentiate between patients with mild disease (METAVIR F0 or F1) versus those with more significant fibrosis (METAVIR F2-F4) could have a widespread clinical utility in managing HCV patients in the future.

The search for the noninvasive marker(s) of liver fibrosis has evolved along two main but quite different approaches: the direct and indirect markers of fibrosis. There are several proposed serum markers; the ideal features of which are:

- Liver specific
- Independent of metabolic alterations
- Easy to perform
- Minimally influenced by urinary and biliary excretion
- Reflective of fibrosis irrespective of cause
- Sensitive enough to discriminate between stages of fibrosis
- Correlate between dynamic changes in fibrogenesis or fibrous resolution

### 1.7 Indirect markers of liver fibrosis

The indirect markers of fibrosis reflect alterations in the hepatic function, but do not directly reflect ECM metabolism. Some of the test panels use general measures, such as age and gender. The first indirect marker of liver fibrosis were transaminases, later associated in the aspartate to alanine aminotransferase ratio (AAR) to detect cirrhosis. The strength of such a marker is its simplicity and availability to every hepatologist and clinician. On the other side, studies showed that its accuracy is highly variable. Other simpler parameters included platelet count, and prothrombin index.
A further evolution was later introduced by Wai, et al, who combined aspartate amiotransferase (AST) with platelet count. The AST to Platelet Ratio Index (APRI) was then assessed in several studies conducted with a cohort of patients with hepatitis C and showed a rather good diagnostic performance and reproducibility, particularly for cirrhosis (AUC range from 0.77 to 0.94). The real strength of such an index is that it is based on blood tests that are routinely performed in patients with liver disease; and with no need for additional blood collection or costs. More recently, APRI has been modified by adding alanine aminotransferase (ALT), and international normalized ratio (INR), with further improvement of the diagnostic accuracy, particularly for cirrhosis.

Another index, the Göteborg University Cirrhosis Index (GUCI), using AST, Prothrombin –INR, and platelet count proved slightly superior for sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), and the area under the receiver operating characteristic (AUROC) curve for prediction of cirrhosis and bridging fibrosis compared with APRI. Although, APRI test is very simple, it is subject to issues related to the reproducibility of AST measurement and platelet count.

Forns and colleagues reported a fibrosis index (Forns’ index) based on platelet count, γ-glutamyl transferase (GGT), and cholesterol levels. It has a rather good NPV of 96% for excluding significant fibrosis, but only 66% PPV for diagnosing significant fibrosis. A study tested the role of APRI and Forns’ index as noninvasive markers in patients co-infected with human immunodeficiency virus (HIV) and HCV. Overall the markers showed lower diagnostic accuracy than in HCV mono-infected patients.

Important concerns about the Forns’ index are: the impact of the serum lipid abnormalities, cholesterol altering medicines, as well as the reproducibility of platelet estimations. However, the most important limit of both APRI and Forns’ index is that they leave almost half of the patients unclassified.

The most widely investigated combination set of noninvasive markers of liver fibrosis is the Fibrotest; a combination of five blood tests based on a mathematical formula: γGT, bilirubin, haptoglobin, apolipoprotein A1, α2 macroglobulin adjusted for gender and age. According to the investigators, for the exclusion of significant fibrosis (METAVIR ≥ F2), it has 100% negative predictive value, and more than 90% positive predictive value, using liver biopsy as a reference. Over all liver biopsy could have been
avoided in 46% of patients on the basis of these findings\textsuperscript{21,130}. On the other hand, it uses two rather uncommon parameters, apolipoprotein A1 and $\alpha$2 macroglobulin, which require precise standardization of laboratory procedures\textsuperscript{131}.

To date, Fibrotest is by far the most investigated and validated noninvasive marker of liver fibrosis with around twenty studies reported in the literature\textsuperscript{24}. The areas under the receiver operating characteristic curve, ROC, for the first year (0.836), and second year (0.870) did not differ ($p=0.44$). With the best index, a high negative predictive value (100% certainty of absence of F2, F3, or F4 fibrosis) was obtained in scores ranging from zero to 0.10 (12% of all patients), and high positive predictive value (>90% certainty of presence of F2, F3, or F4 fibrosis) for scores ranging from 0.60 to 1.00 (34% of all patients)\textsuperscript{130}. The detection of significant fibrosis F2 or greater had a 75% sensitivity and 85% specificity\textsuperscript{130}. The assay performed somewhat better for the assessment of more advanced liver disease (METAVIR stages 3 and 4).

It performed slightly worse when validated by independent workers in chronic HCV (NPV85%, PPV 78%)\textsuperscript{19}, and when validated by the same group, performed similarly less well than in HCV, in patients with chronic HBV with an AUROC of 0.78\textsuperscript{132}. In a study performed in HIV/HCV co-infected patients, Fibrotest performed well, particularly for cirrhosis (AUROC 0.87) that could be excluded with 100% negative predictive value\textsuperscript{133}.

In the Fibropaca study\textsuperscript{134}, an independent prospective multicenter study confirmed the diagnostic value of Fibrotest and Actitest found in the principal study and suggested that both the tests could be an alternative to liver biopsy in most patients with chronic HCV. The Actitest is a modification of the Fibrotest that incorporates ALT along with other biomarkers, and is a measure of the degree of inflammation. The area under the ROC for the diagnosis of activity (A2-A3) was 0.73 (0.69-0.77), for significant fibrosis (F2-F4) was 0.79 (0.75-0.82), and for severe fibrosis (F3-F4) was 0.80 (0.76-0.83). Nevertheless, both the Fibrotest and Acitest bring new insights in the diagnosis of fibrosis in patients with chronic hepatitis C.

A Fibrometer test has been proposed combining platelets, prothrombin index, AST, $\alpha$2 macroglobulin, hyaluronate, urea, and age. The area under the receiving operating characteristic curve (AUROC) for stages F2-F4 was 0.883 for the Fibrometer,
0.808 for the Fibrotest, 0.820 for the Forns’ test, and 0.794 for the APRI. The authors concluding that the Fibrometer has a high diagnostic accuracy for clinically significant fibrosis (F2-F4).

In the same study, the area of fibrosis (AOF) was estimated in viral hepatitis by testing for hyaluronate, $\gamma$-glutamyl transferase, bilirubin, platelets, and apolipoprotein A1. Why use the area of fibrosis? It stems from difficulties in transforming histological stages into a binary variable when staging is evaluated by noninvasive tools. The histological staging usually includes five stages from F0 to F4, the cut off at F2 usually defines clinically significant fibrosis as F2 or higher. Whereas it is well known that the amount of fibrosis varies considerably between stages. Another limitation is the restriction of cirrhosis to one stage (F4), whereas the amount of fibrosis in cirrhosis is four times that of the other four stages.

Because cirrhosis corresponds to only to only one or two stages (depending on the histopathological system used), the range of blood test results for advanced fibrosis in patients with cirrhosis is very limited. For instance in the virus related CLD, the ranges in the Fibrometer test for stages F0-F3 was 0.03-0.99, and for F4 was 0.99-1.00. The authors concluding that the AOF estimation via the blood tests is the only statistically validated quantitative test for the noninvasive diagnosis of fibrosis. However the aim of AOF evaluation is not to distinguish mild F stages due to overlap of AOF values in these stages.

Adams et al, proposed a model (Hepascore) with similar efficacy. It consists of bilirubin, $\gamma$ GT, hyaluronic acid, $\alpha$2 macroglobulin, age and sex and produced AUROC of 0.85, 0.96, and 0.94 for significant fibrosis, advanced fibrosis, and cirrhosis.

Other markers are PGA index; combines prothrombin index, $\gamma$ GT, and apolipoprotein A1, and modified to PGAA index with the addition of $\alpha$2 macroglobulin which resulted in some improvement in its performance.

The Sud score includes age, past alcohol intake, AST, cholesterol, and HOMA-IR, (insulin resistance by the homeostatic model assessment) and performs well with an area under the ROC of 0.77.
1.8 Direct markers of liver fibrosis

The direct markers of liver fibrosis reflect the process of fibrogenesis, the ECM deposition or removal. **Hyaluronic acid** is the most widely studied of the direct markers. It has been studied in hepatitis C, hepatitis B, alcoholic fatty liver disease AFLD, and nonalcoholic fatty liver disease NAFLD. In hepatitis C the AUROC’s have ranged from 0.82-0.92. In hepatitis B the AUROC is 0.98 but requires further validation.

A study by Halfon P, et al, focused on the diagnostic accuracy of HA alone, (instead of combination of markers) in predicting fibrosis and cirrhosis in HCV infected patients. In all 405 patients were studied. Absence of significant fibrosis, severe fibrosis, and cirrhosis can be predicted by HA levels of 16, 25, and 50 µg/l respectively (with NPV of 82%, 89%, and 100% in the same order). Presence of significant fibrosis, severe fibrosis, and cirrhosis can be predicted by HA levels of 121, 160, and 237 µg/l respectively (with PPV of 94%, 100%, and 57% in the same order). Thus serum HA is a clinically useful as a noninvasive marker of liver fibrosis and cirrhosis. It suffers from the need to limit, as much as possible, potential confounding variables such as the effects of exercise and eating.

Another combination panel of matrix markers, **FibroSpect** (hyaluronic acid, TIMP-1, and α2-macroglobulin) has been tested in a cohort of hepatitis C patients, obtaining an AUROC of 0.83 with an accuracy of 75%.

In a recently reported retrospective study, hyaluronic acid, YKL-40, and **FibroSpect II** (comprising hyaluronic acid, TIMP-1 (tissue inhibitor of metalloproteinase 1), and alpha 2 macroglobulin) were assessed with Ishak stages and digital quantification of fibrosis (DQF). Among the serum markers, hyaluronic acid was effective in discriminating Ishak stages 0-1 and Ishak stages 2-3 compared with FS-II, with area under the ROC curve of 0.76 versus 0.66 respectively. All three serum markers predicted advanced fibrosis and cirrhosis. YKL-40 had the highest false positive rates in all categories of fibrosis.

The **SHASTA index** developed by Afdhal N, et al, consists of serum hyaluronic acid, AST, and albumin. A cutoff of less than 0.30 was associated with a
sensitivity of more than 88% and a negative predictive value of more than 94%. The SHASTA index in HIV/HCV has similar accuracy to the Fibrotest and in this study performed significantly better than the APRI test.

Among the glycoproteins, laminin has been assessed as a noninvasive marker mainly for significant liver fibrosis. It showed an overall accuracy of 81% in patients with CLD. Laminin showed good performance, particularly when combined with type IV collagen, with 87% accuracy, 100% specificity and positive predictive value.

**YKL-40** is a recently described glycoprotein that belongs to the chitinase family. It is strongly expressed in the human cartilage and human liver. It is a relatively new marker of hepatic fibrosis and it has only been preliminary evaluated in CLD’s. In patients with chronic hepatitis C, it has an AUROC of 0.81, with 78% sensitivity, and 81% specificity.

Among the collagens, **type IV collagen** has been extensively investigated as a noninvasive marker of liver fibrosis. Its diagnostic performance for significant fibrosis in patients with hepatitis C, has been with an AUROC of 0.83. Several studies evaluated a possible role of procollagen III in patients with hepatitis C and AFLD. In comparative studies conducted in hepatitis C, procollagen III performed less well than type IV collagen and hyaluronic acid.

**Collagenases** and their inhibitors have also been proposed as surrogate markers of liver fibrosis. As noninvasive markers of liver fibrosis, the performance of metalloproteinase 2 (MMP-2) and tissue inhibitor metalloproteinase 1 (TIMP-1) has been high; especially MMP-2 (AUROC 0.97). Unfortunately, it has been difficult to obtain good standardization of the method for routine clinical use. In another study, metalloproteinase 3 (MMP-3) and TIMP-1 had an AUROC of 0.82 for significant fibrosis (METAVIR F2-F4) with 85% specificity.

Measurements of serum cytokines (TGF-β, TNF-β) involved in fibrogenesis have been assessed in a limited number of studies in which the cytokines have shown less value in predicting liver fibrosis compared to the ECM tests.

Several authors have tried to combine different direct markers of liver fibrosis. The **European Liver Fibrosis** study was an international multi-center cohort of 1021 patients with hepatitis C, nonalcoholic fatty liver disease, and alcoholic liver disease.
Discriminant analysis of a test set of samples was used to identify an algorithm combining age, hyaluronic acid, amino-terminal propeptide of type III collagen (PIIINP), and tissue inhibitor of matrix metalloproteinase 1. The sensitivity for the detection of Scheuer stage 3 or 4 fibrosis was 90% at a threshold score of 0.102, and accurately detected the absence of fibrosis (negative predictive value for significant fibrosis, 92%; area under the curve of a receiver operating characteristic plot was 0.804. The algorithm performed equally well in comparison with each of the pathologists. The AUROC was discreet for hepatitis C (0.77), good in NAFLD (0.87), and excellent in AFLD (0.94).

Some of the other techniques are DNA sequence-based protein glycomics, the Glycocirrhotest. The combination of Fibrotest and Glycocirrhotest allows identification of cirrhosis with 100% specificity and 75% sensitivity.

It is thought that increased vascular adhesion molecule-1 (VCAM-1) expression occurs in association with capillarization of the sinusoidal spaces and fibrous septa. In a study of 52 patients with hepatitis with C, soluble VCAM-1 was found to have excellent discriminator power for the detection of advanced fibrosis, sensitivity 100%, and specificity 85%. In this small study measurement of soluble VCAM was of greater diagnostic value than PIIINP.

1.9 Gene Markers and Liver Fibrosis

The complexity of the fibrogenetic process and the high number of factors/cytokines/molecules involved imply that several genetic polymorphisms could influence progression of liver fibrosis. The genetic polymorphisms linked to hepatic fibrogenesis have been investigated mainly in chronic hepatitis C and in alcoholic fatty liver disease. There are many studies that report gene polymorphisms to either favour or reduce fibrogenesis in patients with different forms of chronic liver disease.

One such study, consists of a set of seven marker genes, the cirrhosis risk score (CRS), was found to be a better predictor of high risk versus low risk for cirrhosis in Caucasian patients than clinical factors. The clinical factors such as age, gender, alcohol use, and age at infection, obesity and hepatic steatosis, influence the progression to cirrhosis, but cannot accurately predict the risk of developing cirrhosis in patients with chronic hepatitis C.
The authors validated all significant markers from a genome scan in the training cohort \((n=420)\), and selected 361 markers for the signature building. Subsequently, a signature consisting of 7 markers most predictive for cirrhosis risk in Caucasian patients was developed. The Cirrhosis Risk Score (CRS) was calculated to estimate the risk of developing cirrhosis for each patient.

The area under the receiver operating characteristic (ROC) curve was 0.75 in the training cohort. In the validation cohort, the ROC was only 0.53 for clinical factors, increased to 0.73 for CRS, and 0.76 when CRS and clinical factors were combined. A low CRS cutoff of < 0.50 to identify low risk patients would misclassify only 10.3\% of high risk patients, while a high cut off of > 0.70 to identify high risk patients would misclassify 22.3\% of low risk patients. Thus more importantly fewer of the high risk patients would be misclassified. In conclusion, CRS is a better predictor than clinical factors in differentiating high risk versus low risk for cirrhosis in Caucasian patients.

According to the authors, for the first time, one can estimate the risk of cirrhosis rather than using findings of liver biopsy to project the future course of disease.

### 1.10 Fibrosis Markers to Assess the Effect of Treatment and Disease Progression

According to Afdhal N \(^{19}\), an attractive use of fibrosis markers would be to assess the effects of treatment, and if they can be validated, they could be valuable tools in the search of new antifibrotic treatments. However, none of the currently available markers have yet been validated for use in this way, but several studies have shown that levels of these markers are altered by treatment and they have prognostic value.

According to Afdhal N \(^{19}\), Poynard reported on two studies describing the relationship between response to interferon therapy and the results of Fibrotest performed before and after therapy \(^{157,158}\).

In one of these studies \((n=352)\) \(^{158}\) the authors studied the effect of interferon plus ribavirin on both the Fibrotest and Acititest scores. On follow up, patients who had a sustained virological response to interferon had a substantial reduction in Fibrotest and Acitest scores, as compared to those who were either primary nonresponders or relapsed
following therapy. There was a significant decrease of Fibrotest among the 184 sustained virological responders, from \((0.39 \pm 0.02\) to \(0.28 \pm 0.02\)) at 72 weeks; in comparison with 126 nonresponders and with 42 relapsers.

Furthermore, there was a significant concordance between Fibrotest and fibrosis stage variations. At baseline 32 patients had cirrhosis. Fibrotest scores fell substantially in 17 patients with cirrhosis at baseline and a post treatment reduction of 1 or more stages; from 0.68 to 0.44 for 3 stages improvement, from 0.60 to 0.47 for 2 stages improvement and from 0.61 to 0.56 for 1 stage improvement.\(^{158}\)

These data support the concept that these assays may be useful not only in the initial staging of the live disease, but may also be of value in following the histological response to therapy.

According to Afdhal N, many other studies have evaluated the effect of interferon therapy on levels of fibrosis markers and have compared them to histological findings.\(^{159, 160}\) Most of these studies have shown that serum levels of several of these markers including HA, PIIINP, YKL-40, and TIMP-1 fall in patients who achieve a sustained virological and biochemical response. In these patients, levels continue to fall following treatment and often return to normal levels. In patients who relapse following treatment, the levels frequently fall during therapy but most often return to pretreatment levels with virological and biochemical relapse.

Additionally, treatment with interferon has been associated with a fall in serum markers of fibrosis, independent of a biochemical or virological response.\(^ {161}\) This has been used as evidence that interferon has a beneficial direct antifibrotic effect, presumably through direct inhibition of TGF-β expression.\(^ {162}\)

If noninvasive markers of fibrosis truly reflect fibrogenic activity and the rate of underlying disease progression; then they should have prognostic value: both for predicting clinical outcomes and progression of fibrosis. In this regard the available data are more limited.\(^ {19}\) However several studies have looked at the prognostic value of these fibrosis markers.

Guechot in year 2000 evaluated the predictive value of hylauronic acid in a cohort of 91 patients with hepatitis C associated cirrhosis followed for a median of 38 months. During this time, complications including death, liver transplantation, and severe
complications of cirrhosis occurred in 24 patients. Of all the laboratory tests evaluated, hyaluronic acid was found to have the greatest predictive value and was equivalent to Child-Pugh score\textsuperscript{163}.

In a cohort of 97 patients with primary biliary cirrhosis, Poupon demonstrated that by multivariate analysis HA, PIIINP, bilirubin, and prothrombin time were independently predictive of disease progression\textsuperscript{164}.

According to Afdhal N\textsuperscript{19}, serum TGF-β levels have also been used to assess disease progression\textsuperscript{165,166}. In a 12 month study of 39 patients with hepatitis C infection, who underwent paired liver biopsies, Kanzler showed that those patients who had progressive liver fibrosis had higher levels of serum and tissue expression of TGF-β than those who did not progress\textsuperscript{165}.

Similarly, prognostic value of the Fibrotest was compared with biopsy staging for predicting cirrhosis decompensation and survival in patients with chronic HCV infection. The investigators concluded that the Fibrotest measurement of HCV biomarkers has a 5-year prognostic value similar to that of liver biopsy\textsuperscript{26}. According to the authors, Fibrotest was a better predictor than biopsy staging for HCV complications, with the area under the ROC, 0.96 vs 0.91, respectively; it was also a better predictor for HCV related deaths, AUROC 0.96 vs 0.87 respectively. The prognostic value of Fibrotest was also significant in multivariate analyses after taking into account histology, treatment, alcohol consumption, and HIV coinfection\textsuperscript{26}.

### 1.11 Fibroscan & Caffeine Breath Test

In the noninvasive evaluation of liver fibrosis, a new technology (Fibroscan) that measures liver stiffness has been developed. The rationale has been based on correlation between liver fibrosis and stiffness.

Using a probe (Fibroscan, Echosens, Paris) that includes an ultrasonic transducer, a vibration of low frequency (50 MHz) and amplitude is transmitted into the liver. The vibration wave induces an elastic shear wave that propagates through the organ. The velocity of this wave as it passes through the liver correlates directly with tissue stiffness and by simultaneously using a pulse-echo ultrasound by means of the
same probe, the velocity of the wave is measured. The harder or stiffer the tissue, the faster the shear wave propagates. Results are expressed in kilopascals (kPa). Fibroscan measures liver stiffness of a volume that is approximately a cylinder of 1 cm diameter, and 2 cm long, 100 times greater in size than a standard liver biopsy. Thus it is more representative of the entire hepatic parenchyma $^{167}$.

In a large multicenter study of 327 patients, to investigate the use of liver stiffness measurement in the evaluation of liver fibrosis in patients with chronic hepatitis C, the areas under the receiver operating characteristic curve (ROC); for $F \geq 2$ was 0.79, for $F \geq 3$ was 0.91, and for $F \geq 4$ was 0.97.

The authors concluded that they found significant positive correlation between liver stiffness measurement and fibrosis stages in patients with chronic hepatitis C $^{167}$.

In a prospective study with more than seven hundred patients with CLD’s of different aetiologies, the Fibroscan obtained an AUROC ranging between 0.8 for detecting significant fibrosis to 0.96 for diagnosing cirrhosis $^{168}$. The limitation is that it requires a costly device to measure liver stiffness $^{24}$. Similarly, many of the direct markers of noninvasive evaluation of fibrosis and few of the indirect markers are only available in highly specialized research laboratories and are thus not routinely available.

Combining a Fibroscan with biomarkers may increase the accuracy of both tests $^{169}$.

Caffeine has high oral bioavailability and has almost exclusive hepatic metabolism, principally via demethylation by cytochrome P450 1A2 (CYP1A2). This property renders it an ideal substrate for a quantitative assessment of liver function. In the study, cirrhotic patients were characterized by significantly reduced caffeine breath test (CBT) values (1.15), compared with controls (2.23), and hepatitic patients (1.83). There was significant inverse relationship between the CBT and Child-Pugh score ($r = -0.74$, $p = .002$). The authors concluding that $^{13}$C-CBT represents a valid indicator of plasma caffeine clearance and also correlates reproducibly with hepatic dysfunction $^{170}$. 

1.12 Meta-analysis of Studies of Fibrosis Markers

A meta-analysis of studies of fibrosis markers was published in 2006. It included 14 studies up to the year 2004 with 10 different panels. The discussion includes some of the important differences between studies and future research recommendations:

Although the quality of the included studies in the meta-analysis was assessed using the quality assessment of diagnostic accuracy studies (QUADAS) tool; yet it goes without mentioning that there were important differences:

(1) Whilst all studies included patients from specialist clinics, there was variation between studies in population characteristics, prevalence of severe fibrosis, and methods of test validation and type of markers used, some studies used indirect markers, some direct markers or a combination thereof, some were prospective some retrospective, patient characteristics varied widely between studies; the quality of liver biopsy varied depending on length of the liver biopsy and the number of portal tracts.

(2) Few tests are excellent and perform better than others. Since 2004, there are other works published where the performance of noninvasive markers is even better.

(3) In the well known studies, the number of individual patients correctly classified is higher, more than 40%-50%. In the meta-analysis the reported figure is 35% but at a PPV of 90% and NPV of 95%. Relaxing the probability of making correct assignment can increase this figure.

(4) The prevalence of significant fibrosis varied between studies from 17 to 80% (median 40%). Predictive values of tests are affected by disease prevalence, leading to a lack of generalizability. It is possible some tests might perform better in low or high prevalence populations. For example those with a high sensitivity across lower test scores will perform best in low prevalence populations as the NPV will be higher and the test is applicable to a significant part of study population; the converse would apply in high prevalence populations.
(5) As already discussed, the most important fundamental methodological limitation in assessing noninvasive markers is the use of liver biopsy as a reference standard, and this may contribute to the moderate performance of these tests. The quality of the reference standard is impaired due to the sampling error, length of the biopsy sample, fragmentation, number of portal tracts, and observer variability. Data on the discordant results between histology and one panel of markers has been explored with attribution of discordance to biopsy failure in 18% cases, failure of markers in 2.4% and non-attributable in 8.2% cases. The authors concluding that in many cases of differences it is the shortcomings of the biopsy that are responsible and this leads to an underestimation of the diagnostic performance of the serum markers.

(6) The majority of the proposed biomarker tests—and in particular, those available for use in clinical practice—have an AUC of between 0.80-0.85, not for staging the disease, but for differentiating mild form (F0-F1) from significant fibrosis (F2-F4). Since the biomarker is validated against the biopsy, and the accuracy of the biopsy is only 80%, it is probably statistically impossible for a biomarker to perform any better for staging fibrosis. Interestingly, the performance of biomarkers is superior at the extremes of the disease; the indeterminate results occur when patients have F1-F2 disease. This is not surprising, since these stages are relatively artificial separations of spectrum of a dynamic disease process.
The review of the literature is presented in subsequent chapters on noninvasive evaluation of liver fibrosis under the following headings:

2. Epidemiology of and natural history of hepatitis B and C viruses
3. Hepatic injury, fibrosis, and fibrogenesis (The cell and molecular biology)
4. Liver biopsy and hepatic histopathology
5. Noninvasive evaluation of liver fibrosis
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LITERATURE REVIEW
Chapter 2

EPIDEMIOLOGY

&

NATURAL HISTORY

OF

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2.0 History

Hepatitis B infection, although recognized nearly 40 years ago with the identification of Australia antigen, remains a global health problem. Clinical and epidemiological studies began to differentiate among various types of acute hepatitis in the decades after World War II. The groundbreaking studies of Krugman and colleagues in 1967 firmly established the existence of at least two types of hepatitis, one of which (then called serum hepatitis, and now called hepatitis B) was parenterally transmitted. Serological studies were conducted independently by Prince and colleagues, and by Blumberg et al. Searching for serum protein polymorphisms linked to disease, Blumberg and colleagues identified an antigen (termed Au) in serum from patients with leukemia, leprosy and hepatitis.

By systematically studying patients with transfusion associated hepatitis, Prince and coworkers independently identified an antigen, termed SH, that appeared in the blood of these patients during the incubation period of the disease, and further work established that Au and SH were identical. The antigen represented the HBsAg. These preliminary studies made possible the serologic diagnosis of hepatitis B virus and opened up a whole new world of investigation and research in the field of Hepatology.

2.1 Introduction

It is estimated that 2 billion people worldwide have been infected with HBV, 350 million chronically infected, and 50 million new cases are diagnosed annually. Implementation of HBV vaccination programs has led to significant reductions in the HBV infection rate among children and health care workers. The clinical spectrum of HBV infection is broad, ranging from asymptomatic chronic carriers to fulminant hepatitis with many factors influencing the natural history of the disease: host (including age at the time of infection, gender, immune status); viral characteristics (viral load, genotype, mutation), and exogenous factors such as (viral co-infection, alcohol consumption and chemotherapy). The Hepatitis B e Antigen (HBeAg) negative
chronic hepatitis is increasingly recognized worldwide, which has important implications for therapeutic strategies.\textsuperscript{15}

### 2.2 HBV Disease Burden

HBV is one of the most common infectious diseases in the world. Of the 2 billion people who are infected with HBV, over 350 million are chronically infected, and over 1 million individuals die annually of HBV related chronic liver disease.\textsuperscript{17} 93\% of which are related to cirrhosis and HCC and 7\% from fulminant acute HBV.\textsuperscript{18} Over all cirrhosis, liver failure, or hepatocellular carcinoma develop in 15-40\% of individuals with chronic HBV infection.\textsuperscript{19} In the US, a fourfold increase in age-adjusted death rate due to HBV was noted between 1979 and 1998, most notably in men and non whites, despite the availability of HBV vaccine for the last 20 years.\textsuperscript{20} Hospitalization for HBV related liver disease increased to 4.9 fold along with increases in hospitalizations for HCC and in-hospital deaths. Hospital charges related to HBV also doubled over the 10 year period.\textsuperscript{15} A decision analysis performed by the National Immunization program estimates that if routine infant HBV vaccination with three dose coverage in 90\% and no birth dose, could be achieved; 68\% of HBV related deaths could be prevented.\textsuperscript{18}

### 2.3 The Worldwide Prevalence of HBV

The prevalence of HBV infection varies markedly in different geographic areas of the world, as well as in different population subgroups. The world can be roughly divided into three distinct areas based on Epidemiology. The developed countries of the world fall in low endemic areas, while the developing countries of the world have the high level of endemicity, and other countries falling in between.\textsuperscript{16,17} Broadly speaking, the level of endemicity depends on the socioeconomic development, and access to health care facilities, barring few special population subgroups as exceptions.\textsuperscript{16}

The global distribution of various genotypes is: Genotype D (Pakistan, Afghanistan, Iran and middle east), Genotype B and C (China, south east Asia), Genotype A and D (Europe), Genotype A, B, C, and D (North America), Genotype F
(Central America), Genotype H and F (South America) and Genotype A, E and G (Africa). The HBsAg prevalence is subdivided as low <2%, intermediate 2-8%, and high >8%.

The regional prevalence of HBsAg varies from 0.1% -0.5%(>2%) in low prevalence areas (US, Canada, Northern, Western and Central Europe, Australia & New Zealand), to 2-5% in intermediate prevalence areas (Mediterranean region, Eastern Europe, Russian Federation, Japan, Middle East, Central and South America) to 8-20% in high endemic areas (sub-Saharan Africa, south east Asia: southern China, Taiwan & the Pacific rim).

Chronic hepatitis B infection remains highly endemic in most Asian countries, with prevalence rates as high as 16%, and is a major cause of morbidity and mortality due to complications of cirrhosis and hepatocellular carcinoma (HCC). In the areas of high endemicity, 70-90% of the general population has serologic evidence of current or resolved HBV infection. In these areas, most HBV infection is transmitted perinatally or in early childhood (< 5 years). It is estimated that the perinatal route accounts for 50% of the cases. In the intermediate and low endemicity areas, transmission is more likely to occur in later childhood or adulthood.

In the Asian countries, the prevalence is: 0.8% in Japan, 1.5% in Saudi Arabia, 3.1% Vietnam, 4.6% in China, 2.5%-5% in Indonesia, 7.3% in Korea, >8% in Thailand, 5-16% in Philippines, and >10% in Taiwan.

### 2.4 Transmission of HBV

HBV is ubiquitous in body fluids, including blood, saliva, sweat, breast milk, tears, urine, vaginal secretions, semen, and menstrual blood. Because HBV is resistant to breakdown outside of body, it is easily transmitted through contact with infected body fluids. Viral transmission can be mother to child (vertical or perinatal transmission) or by percutaneous or mucosal exposure to infectious body fluids (horizontal transmission). After infection, the incubation period varies ranges from 45 to 160 days (mean 120).
2.5 Vertical Transmission

Perinatal vertical transmission is the most common route of transmission worldwide. The presence of HBeAg in the mother’s serum is associated with greater infectivity. The risk of perinatal HBV infection among infants born to HBV-infected mothers ranges from 10-40% in HBeAg negative mothers to 70-90% in HBeAg positive mothers. If a pregnant woman contracts acute HBV infection during the first or second trimester, HBV rarely infects the foetus, whereas infection occurring in the third trimester or in the postpartum period will more likely lead to infection in the infant. This observation suggests that infection of infants born to HBV positive mothers most likely occurs in the perinatal period as opposed to in utero, and the risk is associated with maternal replicative status. Wang et al. reported that in 33 HBeAg positive mothers, 70% of their infants had HBeAg positivity at the time of delivery as compared to 0% positivity in 21 infants born to HBeAg negative mothers; suggesting transplacental HBeAg acquisition. Milich et al. had suggested that transplacental HBeAg movement...
may suppress the development of cellular immune response to nucleocapsid proteins that are a major target during immune clearance of infected hepatocytes. However more recent animal data doesn’t support this hypothesis, showing that small amounts of HBeAg transferred via transplacental, lactogenic, or renal routes did not induce tolerance 34.

The concept of perinatal transmission has important practical application. Immediate post natal vaccination with both passive and active immunoprophylaxis for at risk infants born to HBsAg positive mothers significantly reduces the risk of transmission. And HBV vaccination has been introduced in the EPI Programme. But despite this 5%-10% of infants born to HBeAg positive mothers subsequently become HBsAg positive in follow up 35. This may be related to high level of maternal viremia 36, intrauterine infection 37, or HBV mutation in the surface protein 15,38. Anti-viral therapy to suppress HBV vaccination in late pregnancy may reduce vertical transmission 16,39.

Nevertheless, universal infant vaccination has been a success story. For instance, in the United States, vaccination coverage among children aged 19-35 months increased from 16% in 1992 to 90% in 2000 40. From 1990-2002, the incidence of acute hepatitis B decreased by 67% across all age groups, while in children under 20 years, the incidence decreased by 89% 40. The vaccination is recommended for all adults at increased risk of infection (e.g., immunocompromised individuals, health professionals, IV drug users); in cases of chronic infection, vaccination of sexual partners and household contacts is also recommended 27. It can also be offered to adult population at large, to desirous individuals. In the US, vaccination is also recommended for all adolescents 29. Other preventive measures include needle exchange programmes, screening of blood products, and educational approaches 16,39.

2.6 Horizontal Transmission

In areas with a low prevalence of HBV, there is horizontal transmission via sexual contact, injection drug use, or occupational exposure to blood and blood products 11. Percutaneous routes of exposure include transmission of blood or blood products 41,42, contaminated health related equipment and needles 43,44,45, and injection drug use 42,46. Less commonly, tattooing and acupuncture have also been implicated in HBV
transmission. Blood product screening has virtually eliminated this source of HBV transmission; however, in underdeveloped countries, re-use of medical instruments, contaminations of multiple dose medicine vials, and re-use of disposable needles remains as risks for infection. Contamination of dialysis equipment is also a source of transmission if isolation of infected patients and strict adherence to infection control measures are not practiced. Reflecting these lapses, clusters of acute HBV continue to be reported in hemodialysis units. Person to person spread of HBV between household contacts can occur, as HBV can survive in the environment for 7 days or more. Contamination of surfaces with blood or other secretions is the most common source of transmission. Reports among Southeast Asian refugee children showed that 6% to 11% of children born to HBsAg negative mothers were HBsAg positive, indicating probable child-to-child or household transmission.

2.7 Natural History of Chronic Hepatitis B Infection

One of the major determinants of acute HBV infection is age and immune competence at the time of infection. In infants and children, the initial HBV infection is typically subclinical and a large percentage of acute cases proceed to chronic infection. If acquired in adulthood, as is often the case in areas of low endemicity, progression to chronicity is uncommon and symptomatic acute HBV infection is more common.

2.8 Perinatal or Childhood Acquired Infection

With the development of more sensitive molecular assays, a better understanding of the natural history of perinatally acquired HBV infection has been possible. Broadly, four sequential phases of HBV infection can be defined:

1. Immune Tolerance
2. Immune Active/Clearance
3. Nonreplicative
4. Reactivation

In the immune tolerance phase, there is minimal activity against the
virus, viral replication is high, as evidenced by the high serum DNA levels, serum aminotransferase levels are normal, and patients are generally asymptomatic. Histology in this phase shows minimal inflammatory activity. This phase can last for the first two decades of life in the perinatally infected, with a low rate of spontaneous HBsAg clearance\textsuperscript{15,52}.

In the second phase of \textbf{immune activity/clearance}, previously inactive HBV carriers have recurrent episodes of clinical reactivation as immune mediated destruction of infected hepatocytes occurs, leading to elevated liver enzymes and decreased HBV DNA levels. Vertically infected patients often become symptomatic for the first time in this immune active phase, presenting with elevated liver enzymes and HBeAg positivity. They may also seroconvert to anti-HBe positivity sooner. The duration of this second phase is variable from months to years. Reactivation can be severe enough to mimic fulminant acute infection\textsuperscript{15}.

In the third phase, HBV DNA levels have fallen to lower levels, seroconversion from HBeAg positive to HBeAg negative/anti-HBe positive occurs, aminotransferase levels normalize, and histology is again quiescent. This third phase is commonly referred to as \textbf{“inactive carrier”} state. Progression from the active hepatitis seen in the second phase to the seroconversion of HBeAg in the third phase is seen as favourable because it is associated with cessation of viral replication and reduced necro-inflammatory activity with a diminished risk of disease progression \textsuperscript{53,54}. Around 50\% of patients clear HBeAg within 5 years of diagnosis, 70\% within 10 years \textsuperscript{55}. Regression of fibrosis may occur months to years after seroconversion \textsuperscript{15,56}.

The outcome after HBeAg seroconversion depends on the degree of pre-existing liver damage; patients without liver damage may suffer only slight fibrosis or mild chronic hepatitis. Those with pre-existing cirrhosis may experience further complications \textsuperscript{23}. Patients remain HBsAg positive with integration of viral DNA in to the host’s hepatocyte genome \textsuperscript{11}, and with detectable HBV DNA in serum measured by sensitive polymerase chain reaction (PCR) based assays\textsuperscript{2,55}.

With time, some of these patients become HBsAg negative. The annual rate of delayed clearance of HBsAg among patients with chronic HBV infection has been estimated to be 0.5\% to 2\% \textsuperscript{57,58}. HBsAg clearance is more likely to be delayed in female
patients, those who are older, and those with histological evidence of chronic hepatitis and cirrhosis. Despite HBsAg clearance, some patients have residual liver disease, and hepatocellular carcinoma, HCC, may develop. Cryogenic cirrhosis or HBsAg negative HCC may be misdiagnosed in these patients if they are not previously known to be HBsAg positive.

Some infected persons may have the fourth phase. Reactivation can occur spontaneously or under circumstances of immunosuppression and is usually associated with elevated ALT and HBV DNA levels. Reactivation of hepatitis B may be mild and asymptomatic, or it may be severe and lead to fulminant hepatic failure, especially in patients with underlying cirrhosis. In rare instances, reactivation of HBV has been reported after HBsAg seroconversion.

### 2.9 Adult Acquired Infections

In contrast to childhood acquired infection, only 1% to 5% of immune competent adults become chronically infected after acute HBV infection. A much higher percentage (30% to 50%) present with an icteric illness at the time of infection. Fulminant hepatitis occurs in 0.1% to 0.5% of acute HBV cases.

Adult acquired HBV infection in those who are immune compromised due to HIV co-infection can have altered disease progression. In HIV co-infected individuals, spontaneous clearance rates for HBsAg and HBeAg are decreased compared with non HIV infected patients. When followed in a long term study, co-infected individuals had 12% loss of HBeAg at 5 years versus 49% for non HIV infected patients. HIV/HBV co-infection is also associated with lower ALT levels and higher HBV DNA serum levels; indicating more active viral replication in the setting of a depressed immune system unable to mount as vigorous a cytopathic response.
2.10 HBeAg Negative Chronic Hepatitis B Virus
(The Precore and Core Promotor Mutants)

During the second phase of HBV infection (the immune clearance phase), mutations in the core promoter and precore regions may occur that decrease or prevent the synthesis of HBeAg but do not impair viral replication. The host immune response may select these precore and core promotor variants. The most common of the mutants are in which there is a G-to-A change at nucleotide 1896.

The preC-C (precore-core) region encodes hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg). These two proteins are also derived by alternative initiation of translation at two in-frame AUG codons. The internal AUG encodes the 21-kD C protein, the structural polypeptide of the viral capsid, whereas the upstream AUG directs production of the 24-kD preC protein. The preC region encodes a signal sequence, which directs the chain into secretory pathway. As the chains traverse the Golgi complex, cleavage by cellular proteases generates HBeAg, a 16-kD fragment that is secreted into the blood. HBeAg plays no role in viral assembly, and its function is not clear. It is not required for viral replication; mutants bearing chain-terminating lesions within the preC region replicate well in culture, and in fact, arise frequently during natural infection.

Patients who are HBsAg positive (>6 months), anti-HBe positive, HBeAg negative with detectable levels of serum HBV DNA and who have evidence of disease activity (elevated aminotransferases or histological inflammation) have HBeAg-negative chronic hepatitis B. Funk et al, has suggested that HBeAg chronic hepatitis B is more common worldwide than previously reported with a prevalence of 33% in the Mediterranean, 15% in the Asia Pacific, and 14% in the United States and Europe. Chu and the HBV Epidemiologic Study Group have described the presence of the precore variant in 27% and the core promoter in 445 of 694 patients in the United States. Several others also suggest an increasing prevalence of HBeAg–negative chronic hepatitis B.

The reason for this changing epidemiology is still not clear, but may be related to several factors including more widespread treatment of HBeAg positive disease, vaccination, and environmental changes, but further population based studies are
warranted to determine true prevalence rates. Vaccination, by diminishing the acute HBV and subsequent chronic HBV in which HBeAg is typically found during the early phase of infection, may have reduced the prevalence HBeAg positive HBV infection\(^{15}\).

HBeAg negative chronic HBV and its increasing recognition have implications for treatment and disease outcomes. Treatment with interferon and other drugs is more difficult in this group due to a higher rate of relapse after cessation of treatment\(^{77,78,79,80}\).

### 2.11 Vaccination

The first plasma derived vaccine for HBV was introduced in 1981; but it wasn’t until 1986 when a recombinant vaccine was available, and vaccination became widely accepted\(^{15,81}\). As of 2003, 79% of the 192 WHO member states had adopted the policies of universal childhood immunization against HBV\(^ {82,83}\). Thus, despite a safe and efficacious vaccine that has been available for more than 20 years, more global institution of immunization recommendations is needed. Nevertheless the vaccine has dramatically reduced the occurrence of chronic HBV infection and hepatocellular carcinoma; and can thus be considered the first anticancer vaccine\(^ {84}\).

The success of vaccination programs has been clearly demonstrated in Taiwan. There the program was established in 1984, first for the high risk neonates, then extending to cover the children and adults. As a result over all acute infection rates have decreased by 90% (mostly among children and adolescents). The prevalence of chronic HBV infection in children \(< 15\) years old was reduced from 10% to 0.7%, and rates of HCC among children also declined by 50%\(^ {14,81,85}\).

In the United States, due to immunization of all persons up to 18 years of age; the overall incidence of acute HBV infection has reduced from 8.5 cases per 100,000 in 1990, to 2.8 per 100,000 in 2002, a decrease of 67%\(^ {86}\).

### 2.12 Risk Factors for the Progression of Disease

Longitudinal studies in patients chronically infected with HBV indicate that the incidence of cirrhosis ranges from 2-5 per 100 person-years and the 5-year cumulative incidence ranges between 8% and 20%\(^ {87}\). Fattovich et al\(^ {88}\), has reported that HBeAg
negative chronic hepatitis had a higher progression to cirrhosis (8-10 per 100 person-years) than HBeAg positive chronic hepatitis. This may reflect the duration of infection, and a late phase in the natural history of the disease, as opposed to de novo infection with a variant not producing HBeAg. The host and viral factors implicated in disease progression are shown in table 2.  

<table>
<thead>
<tr>
<th>Host factors</th>
<th>Viral factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-infection: HCV, HDV, HIV</td>
<td>Ongoing viral replication, HBV DNA</td>
</tr>
<tr>
<td>Alcohol use</td>
<td>Recurrent episodes of reactivation</td>
</tr>
<tr>
<td>Older age</td>
<td>Possible genotype</td>
</tr>
</tbody>
</table>

Table 2: Host and viral factors in HBV disease progression
EPIDEMIOLOGY AND NATURAL HISTORY OF HCV

2.13 Introduction

We are also facing a pandemic of HCV infection. More than 3 % of the world population, or about 170 million people may be infected with HCV \textsuperscript{89}. The prevalence of HCV is increasing and according to Centers for Disease Control (CDC) estimates of the future burden of chronic hepatitis C predict at least a 4 fold rise in the number of persons with long-standing (20 years or more) infection between 1990 and 2015 \textsuperscript{90}.

2.14 History

Our awareness and understanding of viral hepatitis have increased dramatically during the past three decades, but viral hepatitis is not a new problem \textsuperscript{91}. The descriptions of jaundice exist in the literature as far back as several centuries before Christ and are referenced in the Babylonian Talmud and the writings of Hippocrates \textsuperscript{92}. The infectious nature of the disease was first recognized in the eight century by Pope Zacharis \textsuperscript{93}. However, most of the reports of large population epidemics during the past several centuries probably represent the enteral transmission of what is now known as Hepatitis A. It was not until the practice of inoculation for small pox vaccination was introduced in the 1880’s that the percutaneous route of transmission of the disease was recognized \textsuperscript{94}. Numerous reports of jaundice in patients receiving vaccines or injections for diabetes or syphilis followed during the early 20th century \textsuperscript{95,96,97}. The first association of blood transfusion with the development of hepatitis was reported in 1943 \textsuperscript{98}.

The landmark studies of Krugman and colleagues at the Willowbrook State School in New York documented the transmissibility of hepatitis by human plasma \textsuperscript{99}; and confirmed the long standing clinical observations that both parenteral (serum hepatitis) and enteric (infectious hepatitis) transmission could occur \textsuperscript{3}. Frustrating and largely unsuccessful efforts to identify the specific agents responsible for hepatitis continued for several decades \textsuperscript{100}.

A serologic marker for hepatitis B was first identified by Blumberg et al in 1965 \textsuperscript{1}; although its association with the parenterally transmitted entity known as serum
hepatitis was not recognized until 2 years later. The specific viral agents responsible for hepatitis B and A became recognized during next few years.

These discoveries were major breakthroughs, but it quickly became apparent that neither hepatitis A or B virus could not explain most cases of hepatitis.

The entity of non-A, non-B hepatitis was formally christened by Prince et al, in 1974. An infectious agent was suspected based on the observations that the disease can be transmitted parenterally to the chimpanzees and humans by blood transfusion.

A series of experiments by Bradley and colleagues at the Centers for Disease Control and Prevention (CDC) characterized the nature of the infectious agent. Filtration studies suggested that the agent was between 30 and 50 nm in size. Its infectivity was abolished by chloroform, which suggested the presence of lipid envelop, and also by formalin, heat (100°C for 5 minutes or 60°C for 10 hours), and β propiolactone ultraviolet light. However, the conventional virologic and immunologic techniques of the time failed to isolate the responsible agent.

Scientists at the Chiron Corporation and in Japan used a different tact based on what was then recently described molecular biologic techniques. This was based on Bradley’s work, which suggested that the non-A, non-B agent was a virus. However, because the genomic nature of this putative virus was not known (although a flavivirus-like RNA viral agent was suspected), both DNA and RNA were extracted for cloning from a large volume of infected serum. Following extensive ultracentrifugation, which was sufficient to pellet down the smallest of known infectious agents, total nucleic acid was extracted, and both RNA and DNA were converted to complementary DNA (cDNA). Restriction fragments were cloned into a recombinant bacteriophage vector to form a cDNA library. These phages were then inserted in Escherichia coli capable of transcribing and expressing the encoded peptide, and the resulting products were screened against sera from patients with non-A, non-B hepatitis. The assumption was that sera from infected patients should contain antibody against the agent. After more than 1,000,000 (1 million) clones were screened, 5 clones were found to be reactive. Of these, one clone (5-1-1) was shown to bind not only antibodies in the serum of non-A, non-B hepatitis patients but also those in experimentally infected chimpanzees who appeared to undergo seroconversion several weeks after exposure. Identification of other clones...
with overlapping regions of the viral cDNA allowed these investigators to establish the entire viral genome.

This breakthrough in 1989 led to an explosion of research on the viral agent, now designated as, hepatitis C virus, and its disease, now called, hepatitis C. With the development of antibody based detection systems, HCV was found to be the major cause of non-A, non-B viral hepatitis.

### 2.15 The Epidemiology of HCV

The prevalence of HCV infection throughout the world is ~3%, which corresponds to about 170 million people. In comparison, Diabetes Mellitus, which is a common disorder affecting individuals of all ages, has a worldwide prevalence is about 180 million; and the prevalence of impaired glucose tolerance is estimated at 197 million.

These estimates of HCV in the population are often based on prevalence rates on volunteer blood donors and may therefore underestimate the true population prevalence. For example, in the United States, the prevalence of ant-HCV in the blood donor population is 0.6%, whereas the population prevalence is 1.8%. Nonetheless, these estimates provide some idea of the worldwide pattern of infection. Wasley and Alter suggest that these variations represent variations in the predominant risk factors. For example, in the United States and Europe, the prevalence is low and is concentrated in young males who predominantly acquire infection in early adulthood during injection drug use. By contrast, the infection is most common in older persons in Japan and Italy, which indicates a risk in the distant past. The high prevalence across all age groups in Egypt indicates a common and ongoing risk factor.

Extremely low anti HCV prevalence (0.01%-0.1%) has been reported among blood donors in United Kingdom and Scandinavia; a slightly higher prevalence (0.2%-1%) has been reported from other European countries, North America and Australia, & New Zealand.

The prevalence is 0.01% in blood donors in Northern Ireland, in German population 0.63%, 1.1% in France (randomly selected subjects), Spain 1.2%
voluntary blood donors, Italy, healthy young population in the north 3.2% to 16.2% in the south.

In USA it is 1.8% in National Health and Nutrition Examination Survey 1988-94, to 1.6% in National Health and Nutrition Examination Survey 1999-2002, and Australia 1.1% (pregnant women).

Intermediate prevalence (1.1%-5%) is reported from Eastern Europe, Mediterranean region, South America and Middle East. It is for e.g., in Russia 0.93% (voluntary blood donors), and 7.5% (paid blood donors).

Very high prevalence >10% is reported from Taiwan, south east Asia, countries of sub Saharan Africa and Egypt, for e.g. in Egypt 24.8% in a study and 17% to 26% in other study.

Like HBV, the prevalence of HCV varies widely in the Asian countries. It is 1.1% in Japan, 0.9%-5.3% in Saudi Arabia, 2.3% Indonesia, 5.52% Korea, 5.6% Thailand, 5.2% Philippines, 5.6-16.9% Taiwan, and 20.6% Vietnam (Ho Chi Minh city area).

2.16 The Prevalence of HBV and HCV in Pakistan

Pakistan falls in the intermediate endemicity area for HBV and HCV. There has been paucity of community based epidemiologic work in Pakistan in the general population, other than healthy blood donors. Also there has been lack of representation from across the country. Some of the representative work from different areas of the country is submitted.

2.17 Epidemiological Studies in Pakistan

The earlier studies and studies on high risk patients noted a high prevalence of hepatotropic viruses. For e.g., in elective surgical patients in Lahore, Shirazi et al, reported 8.75% hepatitis BsAg positive.

A large general population based study of 47,538 individuals noted 2.56% prevalence for HBsAg. Although it was a good study, two aspects require special mention: (i) In the study, the predominant proportion were males, 94%, as compared to
females 6%, and (ii) a relatively younger population was studied. In another study, Qasmi et al noted 3% seropositivity for HBsAg in normal individuals of Karachi. Zakariya et al reported a prevalence of 3.2% for HBsAg in healthy male naval recruits. In a recent study of 15,550 young adults seeking recruitment in the Armed Forces, 504 (3.24%) were positive for HBsAg. The presence of HBsAg in young healthy Pakistani population has ranged from 3.0% to 3.5%.

Most of the earlier studies were on healthy blood donors; Zuberi et al, Yousuf et al, Rehman et al reported HBsAg prevalence of 3.1%, 0.99%, and 5% respectively in healthy voluntary blood donors. Important to note that the figure quoted by Zuberi et al corroborates with the present day studies. In a study of 1,03,858 blood donors from northern Pakistan, 3.3% were HBsAg positive. In another study of blood donors from northern part of the country, of 1885 patients, 6.4% were HBsAg positive. In a study from the Multan region, 6000 blood donors were screened. 3.37% tested positive for HBsAg. In Lahore, Bukhari, et al showed 25% prevalence of hepatitis B virus in patients, overall prevalence 5% (1456/29,131). The carrier state in healthy donors was 3.8% (925/27,057).

There are not many studies in Pakistan on the prevalence of pre-core mutant strains. In one of the study from northern Pakistan, HBV DNA was found in 19% (19/100) patients of HBeAg negative and anti-HBe positive carriers suggestive of a prevalence rate of 19% for precore mutants. Raised serum ALT (surrogate marker of liver damage) was found in 42.1% (8/19) carriers with detectable HBV DNA and HBeAg negative (pre-core mutants) as compared to only 11% patients infected with wild virus; indicative of a more detrimental effect on the liver for pre-core mutants during the course of chronic HBV infection.

Similarly, for HCV earlier studies and studies in high risk populations noted a high prevalence. Bhopal et al, have reported anti HCV prevalence of 16.31% in general surgical patients of Rawalpindi, but probably high risk population was studied. Shirazi et al, in a study on elective surgical patients in Lahore, reported 9.24% hepatitis C positive. The results show high seroprevalence of hepatitis B & C in elective surgical patients.
Studies in the general population: a very widely cited study both locally, and in the international literature, on randomly selected subjects in Hafizabad noted anti HCV prevalence of 6.5%\textsuperscript{130,151}. In the large population based study, cited before of 47,538 individuals, the anti HCV prevalence was 5.31%\textsuperscript{136}. Zakariya et al noted a prevalence of 2.2% anti HCV in healthy male naval recruits\textsuperscript{138}. In the recent study on 15,550 healthy subjects, 574 (3.69%) were positive for anti HCV\textsuperscript{139}.

In the study on 1,03,858 blood donors, the prevalence of anti HCV was 4.0%\textsuperscript{145}. In another study on 1885 blood donors, the anti HCV prevalence was 4.7%\textsuperscript{146}. In the study from Multan with 6000 screened blood donors anti HCV was 0.27%\textsuperscript{147}. Similarly in other studies the prevalence of anti HCV in blood donors was reported around 5%\textsuperscript{152}.

Aziz et al, noted amongst health workers of Civil Hospital Karachi, 2.4% prevalence for HBsAg, and 5.6% for anti HCV antibodies. Their results show that the prevalence of antibodies to HCV in health workers was 20 folds higher than health workers in developed countries\textsuperscript{153}. Zuberi et al\textsuperscript{142} and Rehman et al\textsuperscript{144} reported that in health care personnel the prevalence of HBsAg was 2.8% and 5% respectively. In the dental practice the prevalence was 1.66% for HBsAg and 1.26% for HCV antibody\textsuperscript{154}.

Zaidi, et al reported a decreasing trend in the Seroprevalence of viral hepatitis (B & C), in the blood donor population of northern Pakistan. Blood donors screened at H.M.C. Hospital Peshawar showed a seropositivity of 1.40% and 1.34% for hepatitis B & C respectively from 1999-2003. Screening of blood donors from CMH Peshawar during the same period detected 1.75% HBsAg positive and 2.60% anti HCV positive\textsuperscript{155}.

Although some studies have shown a decreasing trend, by far the diseases are epidemic in our country.

Previous studies done in Pakistan have shown that the small pox eradication programs conducted in Pakistan from 1964 to 1982 had given rise to an increased positive serology for anti-HCV. These were noted as 15.9% in Lahore, and 23.8% in Gujranwala\textsuperscript{156}. This could also be attributed to the increased number of injections used in many healthy individuals for minor problems\textsuperscript{139}. A study cited before from Pakistan revealed that more than 10 injections per year in the previous 10 years were far more likely to be associated with increased occurrence of HCV antibodies\textsuperscript{151}. Studies from Southern Italy had shown that individuals who had received Salk Polio vaccine between
In 1956 and 1965 by the multiple uses of unsafe glass syringes may have contracted HCV 157. Like HCV, iatrogenic factors have also been found in the transmission of hepatitis B, which in addition to transfusion of blood and blood products, has been associated with parenteral medications, use of needles, and diagnostic procedures 158.

Because of lack of health facilities in the city slums and rural areas, the risk factors are not addressed to. Unscreened blood transfusions, reuse of syringes and other equipment is rampant. Similarly social customs like ear piercing, tattooing, circumcision and shaving by barbers through use/reuse of unsterilised instruments require attention. As the treatment for these diseases is not affordable by vast majority of people and in case of HCV, a vaccine is not available; mass education of the general population is of paramount importance 130.

2.18 Modes of HCV Transmission

Transfusion of Blood and Blood Products

Historically, transfusion of blood and blood products played an important role in the epidemiology of HCV infection. Although transfusions never accounted for the majority of infections among adults 159. As far back as 1981, two separate American studies showed that there was increased risk of acquiring post-transfusion non-A, non-B hepatitis following blood transfusion, if the donors have elevated ALT levels as compared to normal ALT levels 160,161. In the 1980’s transfusion associated hepatitis accounted for 20% of the cases 113. For e.g., by 1986 the incidence of post transfusion HCV ranged from 5% to 13%, which declined to between 1.5% to 9% from 1986-1990 130. Since 1990, when anti-HCV screening of blood donors became mandatory, the incidence of post transfusion HCV hepatitis declined to <1%, but not completely eliminated. This is because some of the earlier generation ELISA tests are not sensitive enough to pick up early anti-HCV detection 162. Furthermore, seronegative HCV carriers were responsible for 10%-15% of HCV transmission 163. Nucleic acid testing of all donors for HCV RNA has been started in some countries, such as USA, and has reduced this risk even further 163.
Plasma derived products such as clotting factors concentrates frequently were contaminated with HCV until 1987, when manufacturers incorporated effective viral inactivation procedures. Thus haemophiliacs have a very high incidence of HCV infection, 46%–90%. Although viricidal procedures for blood products such as heat treatment, pasteurization, and solvent–detergent treatment have nearly totally eliminated the risk of HCV transmission, they nonetheless do not guarantee complete security. This is because there have been a number of incidences of HCV transmission via intravenous immunoglobulin preparations.

**Transmission in the Health Care Setting**

Unsafe therapeutic injections, contaminated needles and syringes, contaminated surgical instruments are may be the most important source of HCV transmission worldwide. This is why the developing countries have the higher incidence of HCV than the developed countries. A notable example is Egypt, where the HCV prevalence among all age groups is at least fivefold higher than in developed countries. These high rates of infection have been attributed partially to mass schistosomiasis treatment campaigns in the 1960’s and 1970’s, in which medications were administered with reused syringes that had not been adequately disinfected.

Transmission between patients is uncommon in developed countries and generally is associated with inadequate infection control practices, such as breaks in aseptic techniques, contamination of multi-dose vials, or inadequate cleaning of equipment. With current infection control practices, digestive endoscopy is not associated with HCV transmission. Transmission from HCV infected patients to health care workers can occur, primarily through needle stick injuries, which carry a risk of infection of approximately 1.8%.

**Perinatal Transmission**

Transmission to infants born to HCV RNA positive mothers ranges from 4.6% to 10%, but a threshold RNA concentration has not been identified. Most infants do not test HCV RNA positive during the first month of birth, suggesting that infection
occurs at the time of delivery rather than in utero. Because maternal antibody may remain detectable in the uninfected infant for more than 1 year, anti-HCV testing is not recommended before 18 months of age.

**Intrafamilial Transmission**

The prevalence of HCV among sexual and household contacts of chronic hepatitis C patients though varies between 0%-27%; with majority of studies reporting between 0%-11%. However, no conclusive data exists as to the threshold concentration of HCV required to transmit infection. One study showed direct correlation with the duration of marriage (>20 years vs <20 years) and duration of actual exposure to the index patients but not with serum HCV RNA titers. The nonsexual household transmission of HCV infection is speculative, and includes sharing of toothbrushes, dental appliances, razors and nail grooming equipment.

**Injection Drug Use**

It is currently the most common mode of HCV transmission in the developed countries. For instance in the USA; the proportion of acute cases who reported injection drug use increased from 31% in 1994 to 38% in 1999 and to 45% in 2003.

### 2.19 Natural History of Acute HCV Infection

According to Bialek SR, acute infection with HCV is asymptomatic in the majority of individuals. Recent studies have examined early virologic events in acute infection. Four phases of infection are recognized:

1. **Pre-ramp Phase**
   
   During this phase low level viremia is detected.

2. **Ramp-up Phase**

   It occurs between 1 and 2 weeks after exposure. There is exponentially increasing levels of HCV RNA. The estimated doubling time of HCV RNA is 11 to 17 hours.
3. **High Titer Plateau Phase**

It occurs between ramp up and seroconversion, typically lasts 40–60 days\textsuperscript{184}.

4. **Seroconversion**

Antibody development occurs 9 weeks after exposure on average, but cases are described in which antibody development occurred more than 12 months after first detection of viremia\textsuperscript{182}. In persons who achieve long term clearance of HCV following acute infection, HCV RNA becomes undetectable as early as 3 months but can persist for up to 2 years after exposure\textsuperscript{182,186}.

2.20 **Factors Associated with Development of Chronic hepatitis C Virus Infection**

The majority of patients who have acute HCV infection develop chronic infection. Earlier studies in cohorts of blood donors, transfusion recipients, and adults acquiring infection drug use reported chronicity rates of 76% to 86%\textsuperscript{187,188,189}. In contrast, in cohorts of children infected through contaminated blood only 55% to 71% were persistently infected 15 to 20 years later\textsuperscript{190,191}. In cohorts of young women infected with HCV through contaminated anti-D immunoglobulin in Germany and Ireland, the chronicity rate 15 to 20 years after exposure was 55%\textsuperscript{192,193}. Neither size of the inoculum nor mode of HCV acquisition have been linked consistently with likelihood of achieving viral clearance after exposure or severity of chronic disease. The factors most consistently associated with development of chronicity are: age at the time of infection, and immune status at the time of exposure. Whether females are more likely to clear spontaneously than males is unclear\textsuperscript{159}.

Racial differences in rate of spontaneous clearance of HCV are suggested by cohort studies showing higher rates of HCV RNA positivity in anti-HCV positive African Americans and Asian Americans than in White Americans\textsuperscript{117,189,194}. Persons who have compromised immune responses are at higher risk of developing persistent infection following exposure, as shown in studies of HIV infected persons\textsuperscript{189,194}, and transplant recipients acquiring HCV from infected organs\textsuperscript{195,196}. 
2.21 Natural History of Chronic Hepatitis C Virus Infection

According to Bialek, SR\textsuperscript{159}, in persons who have chronic HCV infection, disease progression is variable; and only a proportion of infected patients develop the serious complications, including cirrhosis and hepatocellular carcinoma (HCC). Available studies indicate that cirrhosis develops in 4\% to 24\% of persons after 20 years of infection. These estimates of cirrhosis risk are strongly influenced by the population studied\textsuperscript{197}, and cohort recruitment methods\textsuperscript{198}. Estimates based upon referred patients from liver clinics had a 20 year rate of cirrhosis of 22\% (95\% confidence interval [CI] 18\% –26\%), whereas studies in blood donors and community cohorts were lower, with cirrhosis estimated to occur in 4\% (95\% CI, 1\%-7\%), and 7\% (95\% CI, 4\%-10\%), respectively after 20 years of disease\textsuperscript{197}. Community cohorts probably provide the most accurate estimates of HCV disease progression in the general population of HCV infected individuals\textsuperscript{159}.

Most estimates of disease progression use cross sectional data in persons who have HCV associated liver disease. Prospective evaluation of sequential biopsies in persons who have chronic infection is a more accurate method to assess progression and risk of complications\textsuperscript{159}. In paired biopsy studies, fibrosis progression of at least one fibrosis stage was shown in 27\% to 41\% with average follow up periods of 2.2 to 6.5 years (table 4, given at the end). These data support the practice of monitoring for fibrosis progression with repeat biopsies every 3 to 5 years\textsuperscript{159}. And here comes the role of noninvasive evaluation of liver fibrosis; to avoid repeat biopsies, and for more frequent monitoring of hepatic fibrosis.

Among European cohorts of patients who have HCV associated cirrhosis, the rate of developing decompensated liver disease is approximately 4\% per year\textsuperscript{199,200}. North American cohorts of patients who have chronic HCV infection and cirrhosis have not been studied. In those who have advanced fibrosis, in particular, cirrhosis, hepatocellular carcinoma (HCC) develops at the rate of 1\% to 7\% (median 3\%) per year\textsuperscript{199-202}. HCV infection has emerged as one of the most common causes of liver cancer in the United States. A study has shown that among persons aged 65 years or older who had HCC the
proportion who had HCV increased from 11% in the period from 1993 to 1996 to 21% from 1996 to 1999.

The majority of natural history studies published have follow up periods of up to 2 decades, but rarely beyond. Outcomes beyond 20 years of infection are largely unknown and are based primarily on modeling. This lack of information is a significant limitation in counseling patients who may have been infected for longer periods; and it also points to the importance of on-going follow up of established cohorts to allow revised estimates of disease progression.

2.22 Factors Linked with Progression of Disease

The host, viral, and environmental factors most consistently associated with progressive fibrosis and the development of cirrhosis include: age at infection, duration of infection, heavy alcohol use, and HIV infection (table 3).

Co-existent liver diseases, such as chronic hepatitis B infection, and most recently, schistosomiasis, seem to be associated with more severe fibrosis than seen in patients who have HCV mono-infection. An important emerging risk factor for fibrosis progression and cirrhosis is steatosis and factors related to metabolic syndrome (body mass index or obesity).

Factors linked with fibrosis progression in some but not all studies include HCV viral load, HCV genotype and quasispecies, and daily cannabis use. There is an increasing focus on the effect of host genetics on fibrosis progression. Specific HLA class I alleles have not been consistently linked with fibrosis progression. HLA class II allelic variation has been linked with severity of disease in studies from Asia and Europe, but further confirmatory studies are needed. Studies of the relationship between fibrosis progression and the genes encoding inflammatory mediators and cytokines, as well as the enzymes involved in oxidative stress, lipid metabolism, and the mutation in the hemachromatosis gene, have been examined, but consistent associations have not emerged.
<table>
<thead>
<tr>
<th>Type of factor</th>
<th>Well established factors</th>
<th>Emerging risk factors</th>
<th>More controversial risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host</td>
<td>Age at infection</td>
<td>Insulin resistance</td>
<td>Race</td>
</tr>
<tr>
<td></td>
<td>Duration of infection</td>
<td>Steatosis</td>
<td>Baseline necroinflammation</td>
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<tr>
<td></td>
<td>Male Gender</td>
<td>HLA Class II</td>
<td>Other genetic polymorphisms</td>
</tr>
<tr>
<td></td>
<td>Elevated ALT</td>
<td>polymorphisms</td>
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<td></td>
<td>Baseline fibrosis</td>
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<tr>
<td>Viral</td>
<td>HBV infection</td>
<td>HCV viral load</td>
<td>HCV genotype</td>
</tr>
<tr>
<td></td>
<td>HIV infection</td>
<td></td>
<td>Viral quasispecies</td>
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<td>Heavy alcohol use</td>
<td>Schistosomiasis</td>
<td>Smoking</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cannabis use</td>
</tr>
</tbody>
</table>

Table 3: Factors associated with advanced fibrosis among patients with chronic HCV infection.\(^{159}\)

**Age at Infection**

Both age and age at the time of infection are associated with risk of cirrhosis development\(^{216,217}\). Individuals exposed to HCV infection at a younger age (<40 years) progress less rapidly than persons exposed at an older age. Additionally, ageing is likely to be an important factor in disease progression. The capacity of an ageing liver to endure ongoing chronic injury may be less than that of a young liver. Thus fibrosis progression is less likely to be linear, and some models suggest that risk of fibrosis progression increases with age\(^{218}\). However, the effect of age on risk of progression may be overestimated by a failure to account for the competing risks related to deaths from natural causes\(^{219}\).

**Gender**

Male gender is consistently associated with higher risk of fibrosis progression (~2.5 fold), and higher rates of cirrhosis and HCC than seen in females. The effect is independent of alcohol consumption, higher body mass index, and iron overload.
Recent retrospective studies suggest a protective effect of estrogens. In one study of 157 women who had chronic HCV infection, the estimated rate of fibrosis progression was higher and histologic activity was higher in postmenopausal and nulliparous women than in premenopausal women who had had prior pregnancies. Additionally, among postmenopausal women, the estimated rate of fibrosis progression was lower in women who received hormone replacement therapy. Other studies of women who had chronic HCV infection report that estrogen exposure, defined by duration of menses, number of pregnancies, and use of hormone replacement therapy, is associated with a reduced risk of HCC. The exact mechanisms by which estrogens may slow fibrosis progression and modify the risk of liver-related complications have not been defined.

**Alcohol Consumption**

Heavy alcohol consumption is clearly associated with progression of fibrosis and cirrhosis in patients with chronic HCV infection. A recent meta-analysis of 20 studies including 15,000 persons chronically infected with HCV, reported a pooled relative risk of cirrhosis associated with heavy alcohol intake of, 2.33 (95% CI 1.67-3.26). Even among HCV infected patients with persistently normal serum transaminases, heavy alcohol use is predictive of more severe fibrosis. Studies indicate that at levels of 50g/day or higher, the effects of HCV and alcohol are additive, but at very high levels (125g/day or more), the effects are synergistic.

**HIV Co-infection**

Among persons who have chronic HCV infection, HIV co-infection is associated with more advanced stages of fibrosis and higher risk of cirrhosis. In a French study, the median time to cirrhosis was estimated to be 26 years (95% CI, 22-34) in HIV-HCV co-infected patients; versus 38 years (95% CI, 32-47) in HCV mono-infected patients. Very similar results were obtained in a study of injection drug users in the United Kingdom (73% male); the median rate of fibrosis progression was 0.17 units/yr in HIV-HCV co-infected patients and 0.13 units/yr in HCV mono-infected patients (p =
0.01). This equated to an estimated time from HCV infection to cirrhosis of 23 and 32 years respectively.226

**Steatosis**

Steatosis is a common histologic finding in patients who have chronic HCV disease (40%-86%), with the majority of patients having mild steatosis (≤ 30% of hepatocytes affected).228,229 The underlying mechanism for steatosis differs by the genotype. Steatosis in persons, who have genotype 3 HCV infection is viral associated. In these individuals, steatosis is best correlated with HCV RNA titers; and steatosis is improved by attainment of sustained virologic response with antiviral therapy.230-234 In vitro studies and transgenic mouse models suggest HCV core protein may play a role in lipid accumulation within hepatocytes.235,236

In contrast, steatosis in persons infected with HCV genotype 1 (and probably all non-3 genotypes) is associated with the same risk factors as seen in non-HCV infected persons who have fatty liver. Coexistent obesity, diabetes, and hyperlipidemia, as well as excessive alcohol use, have been linked with the presence of steatosis in HCV infected individuals.228,229,233,237 In studies assessing insulin resistance, steatosis and insulin resistance are significantly correlated in persons who have non-genotype 3 HCV infection.233,237

Of importance is: whether steatosis causes worsening of fibrosis. Several studies have identified a strong association between steatosis and fibrosis.232,234,238,239 In the largest study, a multinational study of 3068 persons who had chronic HCV infection, independent predictors of fibrosis were inflammatory activity, steatosis, male sex, and older age.234 It remains to be proven whether this association is caused by steatosis per se or by the metabolic factor promoting steatosis or the associated necroinflammation. If steatosis is the ‘cause’ of liver fibrosis, then potential interventions to reduce steatosis may positively influence fibrosis progression. In paired biopsy studies, more progressive fibrosis is seen in patients who have steatosis at baseline or worsening of steatosis during follow up.240,241 Whether steatosis is an independent risk factor for HCC is less clear.159
Published Studies of paired liver biopsy assessment of fibrosis progression in persons with chronic HCV infection

<table>
<thead>
<tr>
<th>Author, year, ref.</th>
<th>N</th>
<th>Baseline characteristics</th>
<th>Interval between biopsies</th>
<th>% with progression</th>
<th>Predictors of progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collier, 2005 JDC’05 [242]</td>
<td>105</td>
<td>68% male, avg age 40 yr; 70% IDU; 86% increased ALT</td>
<td>3.4 year mean (range 0.4-15.3)</td>
<td>26.7% progressed ≥ 1 fibrosis unit (scale to 5); 5% progressed ≥ 2 fibrosis units</td>
<td>Current alcohol use</td>
</tr>
<tr>
<td>Ryder, 2004 SR’04 [243]</td>
<td>214</td>
<td>59% male; median age 1st biopsy 36 yr; 52% IDU as risk factor</td>
<td>2.5 median (range, 1.9-9.4)</td>
<td>32.7% progressed ≥ 1 fibrosis unit (scale to 6)</td>
<td>Age at biopsy; fibrosis score at baseline</td>
</tr>
<tr>
<td>Wilson, 2006 LEW’06 [207]</td>
<td>121</td>
<td>82% male; median age 42 yr; 92% African Americans; 100% IDU; 27% HIV infected</td>
<td>4.2 yr median (range 2.8-6.0); 63% F0 or F1 at baseline</td>
<td>21% progressed ≥ 2 fibrosis unit; rate = 0.04 fibrosis unit /yr (scale to 6)</td>
<td>HCV viral load; ALT ≥ 60 IU/mL</td>
</tr>
<tr>
<td>Wali, 1999 MW’99 [244]</td>
<td>46</td>
<td>Mean age 37 yr</td>
<td>2.2 yr mean; median at baseline F0 (range, 0-2)</td>
<td>41% progressed ≥ 1 fibrosis score; rate = 0.15 fibrosis units/yr (scale to 4)</td>
<td>ALT elevation</td>
</tr>
<tr>
<td>Colleta, 2005 CC’05 [245]</td>
<td>40</td>
<td>45% male; median age 43 yr; ALT persistently ≤ 1.2 ULN</td>
<td>6.5 yr median; 98% F0 or F1 at baseline</td>
<td>35.7% progressed ≥ 1 fibrosis score</td>
<td>High HCV viral load; past alcohol intake &gt; 20 g/d</td>
</tr>
</tbody>
</table>

**Table 4**

Abbreviations: ALT, alanine aminotransferase; F0, fibrosis stage 0, F1, fibrosis stage 1; HCV, hepatitis C virus; IDU, injection drug user; ULN, upper limit of normal
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Chapter 3

HEPATIC INJURY
FIBROSIS
AND
FIBROGENESIS

THE CELL AND MOLECULAR BIOLOGY
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3.1 Introduction

Fibrosis is a reversible wound healing process that occurs in almost all patients with chronic liver injury. It is the response to various insults, such as viral agents, alcohol, ischemia, medications and hepatotoxins. Fibrosis and cirrhosis may ensue after any of the multiple types of liver injury (Fig. 1&2). The major etiologies of cirrhosis include: chronic viral hepatitis (B or C), alcohol abuse, autoimmune hepatitis, hemochromatosis, Wilson’s disease (copper overload), α-1-antitrypsin disease, recurrent injury to the bile ducts (primary biliary cirrhosis, and primary sclerosing cholangitis), and perhaps congenital lesions. Broadly speaking, the causes of cirrhosis are multiple, and include many congenital, metabolic, inflammatory, and toxic liver diseases (table 1).

Regardless of the etiologic basis, with chronic injury and fibrosis, the clinical outcome is similar, liver architecture and metabolism is disrupted, eventually manifesting as cirrhosis and its complications. Which are: portal hypertension, ascites, encephalopathy, varices, synthetic dysfunction, and impaired metabolic capacity. Thus fibrosis is deleterious both by its direct effects on cellular function and by its mechanical contribution to increased portal resistance.

Although the wound healing response often begins with injury to the hepatocyte, the overall response extends far beyond this event. The injury to the hepatocytes results in a cascade of events. These events include activation and mobilization of a variety of inflammatory cells that release cytokines that not only lead to amplification of the overall response, but also contribute directly or indirectly to the “activation” of the effector cells, usually of the mesenchymal lineage. The typical effector cells are hepatic stellate cells (HSC’s; Fig. 3) and Kupffer cells.

A brief outline is: the hepatocyte damage will cause release of cytokines and other soluble factors by the Kupffer cells and other cell types in the liver. These factors lead to the activation of hepatic stellate cells, HSC, which synthesize large amounts of extracellular matrix components. The activation of hepatic stellate cells involve the transdifferentiation from a quiescent state into myofibroblast-like cells with the appearance of smooth muscle α-actin and loss of vitamin A storage. Once the effector cells (HSC’s) are activated, cytokines (and biologically active peptides) are further
released from the effector cells themselves, creating an autocrine loop, which further amplifies the response (Fig. 5). An important step in this response is the release of matrix degrading proteases and their regulation by specific inhibitors and plasma proteins.  

According to Rockey DC, no matter what the cause of liver injury, increased production of extracellular matrix constituents is the key in all forms of hepatic fibrogenesis. The most prominent and abundant extracellular matrix types include interstitial collagens, types I, and III. Quantitative and qualitative changes in many other matrix components have been described, including proteoglycans, and matrix glycoproteins, such as laminin, fibronectin, and tenascin. Specific changes in matrix composition are similar in all forms of liver injury and hepatic fibrogenesis; which suggests that the general mechanisms of fibrosis are similar.  

Discoveries during the past 2 decades have begun to clarify mechanisms of fibrogenesis and have thereby indicated areas of potential therapeutic intervention. Thus new therapies for hepatic fibrogenesis will be based on fundamental understanding of the basic mechanisms involved rather than on empiric observations. In addition to treating the underlying etiology, such as specific antiviral therapy; of lately, it has been encouraging that novel strategies are being developed to directly address hepatic injury and fibrosis at the subcellular and molecular levels.  

With new understanding and insights into the pathogenesis of hepatocellular damage and hepatic fibrogenesis, key steps such as signaling, activation and gene expression in specific cell types of liver have been targeted by molecular modalities. The state of the art techniques such as: new viral or non-viral vector systems, RNAi, ribosome, and antisense technologies have made it possible to modulate the expression of specific genes involved in the key pathways of liver injury and fibrosis. Gene therapy is still in its infancy, and ideal gene delivery systems for selective gene transfer with high and prolonged gene expression, as well as, less cytotoxicity or immunogeticity remain to be developed. Nevertheless, molecular therapeutics has already proven to be a powerful research tool and explorative experimental medicine. They have demonstrated some promise as novel modalities in reducing liver injury, inhibiting HSC activation, and promoting the resolution of fibrosis.
Although these progresses in treatment of liver injury and fibrogenesis are encouraging, one issue that requires additional attention remains that of targeting these molecular therapeutics to specific cell types (hepatocytes, Kupffer cells or HSC), is critical in avoiding undesired effects on other organs or cell types\textsuperscript{2,20-23}. 

This chapter intends to review the exciting new developments that have been made towards unraveling the cellular and molecular basis of hepatic injury and fibrosis, and partly deals with how the newly acquired insights are leading towards advances in the diagnosis and treatment of chronic liver disease.

### Table 5. Causes of Fibrosis and Cirrhosis\textsuperscript{1}

<table>
<thead>
<tr>
<th>1. Presinusoidal fibrosis</th>
<th>E. Metabolic/Genetic diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schistosomiiasis</td>
<td>Wilson’s Disease</td>
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<td>Idiopathic portal fibrosis</td>
<td>Genetic hemochromatosis</td>
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<td>α1-Antitrypsin deficiency</td>
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<table>
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<tr>
<th>2. Parenchymal fibrosis</th>
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<tbody>
<tr>
<td><strong>A. Infections</strong></td>
<td></td>
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<tr>
<td>Chronic hepatitis B,C,D</td>
<td>Lipid metabolism disorders</td>
</tr>
<tr>
<td>Brucellosis</td>
<td>Urea cycle disorders</td>
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<tr>
<td>Echinococcosis</td>
<td>Porphyria</td>
</tr>
<tr>
<td>Congenital or Tertiary Syphilis</td>
<td>Amino acid metabolism disorders</td>
</tr>
<tr>
<td></td>
<td>Bile acid disorders</td>
</tr>
</tbody>
</table>

| **B. Drugs and Toxins**                       |                                                                 |
| Alcohol                                       | Primary biliary cirrhosis                                           |
| Paracetamol                                   | Secondary biliary cirrhosis                                         |
| INH, Rifampicin, PZA                          | Cystic fibrosis                                                    |
| Amiodarone                                    | Biliary atresia/neonatal hepatitis                                 |
| Methotrexate                                  | Congenital biliary cysts                                           |
| Vit A                                         |                                                                  |
| α-Methyldopa                                  |                                                                  |

| **C. Autoimmune Disease**                     |                                                                 |
| Autoimmune hepatitis                          |                                                                  |

| **D. Vascular Abnormalities**                 |                                                                 |
| Chronic passive congestion                    |                                                                  |
| Hereditary hemorrhagic telangiectasia         |                                                                  |

| **3. Postsinusoidal fibrosis**                |                                                                 |
|                                              |                                                                  |
|                                              | Venoeocclusive disease                                           |
3.2 Biological Principals of Hepatic Fibrosis

According to Friedman SL and Rockey DC several discoveries have played a critical role in understanding the pathophysiologic basis of hepatic fibrogenesis and cirrhosis. These are discussed below:

i) **Hepatic fibrosis initially is a wound healing response** in which damaged regions are encapsulated by extracellular matrix, (ECM) or scar. In all circumstances, the characterization of the fibrotic scar is similar; specifically the extracellular matrix proteins that make it up. The cells and soluble factors participating in this response in liver are similar to those seen in parenchymal injury to the kidney, lung, and skin. This understanding has helped to identify underlying mechanisms and may likely lead to new therapies for fibrotic diseases of many organs, including liver.

ii) **The role of cellular elements in extracellular matrix production.** The discoveries have led to the identification of cellular elements that are responsible for extracellular matrix production and in defining how they respond to injury. One of the most important cellular elements has been the hepatic stellate cells.

iii) **The same cell type produces hepatic fibrosis regardless of the underlying cause.** The activated hepatic stellate cell has emerged as the key cellular source of hepatic scar. How stellate cells become activated in response to a large variety of hepatic insults – from inborn metabolic defects to chronic viral hepatitis – remains largely unknown.

iv) **Soluble factors (cytokines / peptides) and cell-matrix interactions are important mediators in wound response;** certain soluble factors such as cytokines and peptides, as well as extracellular matrix (including interactions with integrins as important receptors mediating cell-matrix interactions) play a critical role in wound response.

v) **The fibrotic scar, and ECM are biologically dynamic milieu, not inert ground substances.** It has been recognized that the fibrogenic process, ECM and the fibrotic scar
can be dynamically modulated (i.e., reversible) by way of the effect of matrix degrading proteases and apoptosis of the effector cells. 

**iii) Hepatic fibrosis follows chronic but not self-limited injury.** Scarring does not develop in patients who survive fulminant hepatitis, despite an abundance of fibrogenic stimuli, unless chronic injury follows. The reason for this observation is not understood, but identifying the factors that make fibrosis reversible may provide important clues to the pathogenesis of fibrosis and cirrhosis.

**iv) Fibrosis occurs earliest in regions where injury is most severe,** especially in chronic inflammatory liver disease resulting from alcohol abuse or viral infection.

### 3.3 Morphological Basis of Liver Fibrosis

Fibrogenesis is a ubiquitous biological process observed in every human chronic inflammatory disease; such as inflammatory diseases of the lung, kidney, skin, or gastrointestinal tract. 

**Fibrosis** is the deposition of extracellular matrix (ECM) components within the liver parenchyma. 

**Fibrogenesis** is the consequence of a dynamic mechanism of gene transcription and protein synthesis of ECM compounds.

For instance, in chronic hepatitis C, liver fibrogenesis develops initially around the portal tract leading to periportal stellate fibrous deposits and expansion (periportal or zone 1 fibrosis). When this process progresses, fibrous tissue extends to the neighboring mesenchymal structures with the formation of fibrous septa. It gradually extends out into the lobules towards the central veins (zone 3) with septa formation. Finally, when most of the portal tracts or central veins are interconnected, bridging fibrosis develops, and annular fibrosis surrounds nodules of the liver cells, and cirrhosis develops.
The fibrous tissue deposition within the liver has several deleterious direct consequences. It reduces the mass of functional parenchyma (liver cells) and disturbs liver architecture and vascularisation\textsuperscript{25}.

### 3.4 Initiation of Hepatic Fibrogenesis – The Cellular Apoptosis

According to Prosser CC \textsuperscript{2}, chronic hepatocellular death via necrosis and/or apoptosis initiates inflammatory responses and hepatic fibrogenesis. Ideally, hepatocytes regenerate and replace dying cells. However, the hepatocellular regeneration in chronic liver injury is often inhibited due to the imbalance of growth factors and the distortion of liver architecture and circulation. Cellular debris and apoptotic bodies accumulate and initiate inflammatory responses, which may form a self-amplifying loop that further compromises recovery from injury, and facilitates the fibrogenic process\textsuperscript{29}.

HSC may engulf the apoptic bodies, which has been demonstrated in vitro, and phagocytosis of apoptic bodies by quiescent HSC facilitates the phenotypic transdifferentiation to myofibroblasts\textsuperscript{30}. Activated HSC with engulfed apoptic bodies were identified in a rat model of bile-duct ligation and liver biopsy specimens from HCV infection\textsuperscript{2,30,31}.

There are two pathways of cellular apoptosis:

#### i) Extrinsic Pathway of Cellular Apoptosis

According to Prosser CC \textsuperscript{3}, this extrinsic pathway is signaled through cell surface death receptors\textsuperscript{32}, including Fas (also known CD95), TNF-\textalpha-receptor-1, and TNF-\textalpha-related-apoptosis-inducing-ligand receptors 1 & 2 (TRAIL-R1 and R2). Tumor necrosis factor-\textalpha (TNF-\textalpha) via TNF-receptor-1 can initiate apoptosis\textsuperscript{32,33}. Examples of this extrinsic pathway of programmed cell death include autoimmune hepatitis, viral hepatitis\textsuperscript{34,35}, chronic alcohol consumption, D-galactosamine (GaIN) plus lipopolysaccharide (LPS) induced liver injury, and ischemia/reperfusion associated liver injury. In these forms of
injury, cytotoxic cytokines or chemokines play a crucial role in the mediation of the injury process \(^2,36-42\).

ii) **Intrinsic Pathway of Cellular Apoptosis**

In contrast to the extrinsic apoptotic pathway, the intrinsic pathway is based on damage or dysfunction of intracellular organelles, such as lysosomes, endoplasmic reticulum, nucleus, and mitochondria \(^43\). This mechanism involves changes in membrane permeability and integrity of the subcellular organelles. Examples are: damage to nuclear or mitochondrial DNA can initiate apoptosis, and the release of cytochrome C from mitochondria triggers apoptotic cascades. The intrinsic pathway of programmed cell death is often seen in drug toxicity or hepatotoxin-induced liver injury, such as acetaminophen overdose, alcohol toxicity, etc \(^42,44\).

Inhibiting apoptosis will alleviate liver injury, and in turn delay or stop the progression of hepatic fibrosis \(^2\).

3.5 **Cellular Sources of Extracellular Matrix - Normal and Fibrotic Liver**

The clear identification of the cellular sources of extracellular matrix in hepatic fibrosis is a significant advance. These are: 1) hepatic stellate cells, (HSC), 2) myofibroblasts (MF) or portal fibroblasts, and 3) sinusoidal endothelial cells \(^1\).

i) **Hepatic Stellate Cells**

Extensive investigation during the past 15 years has helped establish the critical role of hepatic stellate cells in hepatic fibrogenesis \(^2\). The identification and isolation of hepatic stellate cells was a major advance in the understanding of the pathogenesis of hepatic fibrogenesis because it has allowed careful characterization of their biology \(^45\).

The hepatic stellate cell (previously called lipocyte, Ito, fat-storing, or perisinusoidal cell) is the primary source of ECM in normal and fibrotic liver (Fig. 3). Minor contributions by portal fibroblasts are likely \(^1,2\).
Hepatic stellate cells are resident perisinusoidal cells in the subendothelial space between the hepatocytes and sinusoidal endothelial cells. They are the primary site for storing retinoids (vitamin A metabolites) within the body. Stellate cells can be recognized by their Vit A autoflourescence, perisinusoidal orientation, and expression of the cytoskeletal proteins, desmin, and glial acidic fibrillary protein. In strict terms, “stellate cells” may represent a heterogenous population of mesenchymal cells with respect to cytoskeletal phenotype, vitamin A content, and localization. But collectively they are the key fibrogenic cell type in the liver. Stellate cells with fibrogenic potential are not confined to liver; for example they have been identified in pancreas also.

Studies in situ in both animals and humans with progressive injury have defined a series of changes within stellate cells that collectively are termed activation (see below). In this event, a quiescent resting vitamin A-rich cell is transformed into a proliferative, highly fibrogenic and contractile cell characterized morphologically by enlargement of the rough endoplasmic reticulum, diminution of the vitamin A droplets, ruffling of the nuclear membrane, the appearance of the contractile filaments, and proliferation. Cells with features of both quiescent and activated cells are often called transitional cells.

Although simple in concept, the activation process is remarkably complex and involves several important cellular changes. Although it is appreciated that several factors which are prominent in the injured liver are important in stimulation activation (cytokines, chemokines, extracellular matrix), recent data suggest that a much broader range of factors contribute to activation. For example, apoptotic fragments that are derived from hepatocytes seem to stimulate fibrogenesis in cultured stellate cells. Further, hepatitis C virus (HCV) core and nonstructural (NS3-NS5) proteins directly interact with stellate cells and facilitate many features of stellate cell activation (e.g., proliferation, secretion of biologically active transforming growth factor, TGF-β1 and expression of procollagen α1.

Perhaps the most prominent feature of activation is the enhanced production of extracellular matrix. Further, the available evidence now indicates that the overall increase in extracellular matrix deposition that is typical of cirrhosis largely can be ascribed to excess production by stellate cells. Activation is also associated with
other important events, including proliferation $^{55,56}$, contractility $^{57}$, release of proinflammatory cytokines $^{58,59}$ and release of matrix-degrading enzymes and their inhibitors $^{60,61}$.

As noted above, the proliferation of stellate cells occurs in the region of greatest injury. It is typically preceded by an influx of inflammatory cells and associated with the subsequent accumulation of extracellular matrix $^1$.

Stellate cells have now been characterized in many human liver diseases. Alcoholic liver disease is the best studied example $^{62}$. Activation may even occur in the presence of steatosis alone without inflammation $^{63}$. Activated stellate cells have also been observed in viral hepatitis and massive hepatic necrosis $^{64}$. Stellate cells have been characterized in a number of other human conditions $^{62}$, including vascular disease $^{65}$, hematologic malignancy, biliary disease, mucopolysaccharidosis, acetaminophen overdose, leishmaniasis, allograft rejection, and in drug users $^1$. In hepatocellular carcinoma, activated stellate cells contribute to the deposition of tumor stroma $^{66}$.

ii) Sinusoidal Endothelial Cell

Extracellular matrix production by sinusoidal endothelial cells, although less than that by the stellate cells, is nonetheless an important component of early fibrosis. Like stellate cells, this cell type demonstrates considerable heterogeneity in normal and fibrotic liver $^{49}$. Endothelial cells from normal liver produce type III $^{15}$, and type IV collagens $^{67}$, laminin $^{15}$, syndecans $^{15}$, and fibronectin $^{16}$. Following acute liver injury, an increased expression of cellular isoforms of fibronectin by these cells is a key early event because their appearance creates a microenvironment that activates stellate cells $^1$.

3.6 Stellate Cell Activation – The Central Event in Hepatic Fibrogenesis

According to Friedman SL $^1$, hepatic stellate cells are the major source of ECM, and are the key players in liver fibrogenesis and remodeling. Activation consists of two major phases; 1) *initiation* (also called the *pre-inflammatory stage*), and *perpetuation* $^9$, although both are associated tightly $^{68}$. These are finely tuned mechanisms regulated by
several molecules including chemokines and growth factors organized into a complex network (Fig. 6)\textsuperscript{69}.

Initiation represents early stages in gene expression and phenotype that renders the cells responsive to other cytokines and stimuli. Perpetuation represents the effects of these stimuli to maintain the activated phenotype and generate fibrosis. Initiation is largely a paracrine function, whereas perpetuation involves both autocrine and paracrine pathways\textsuperscript{1}.

Upon activation, HSC’s change their phenotype and function into a myofibroblast like cell; a specialized cell type involved in ECM production. In parallel to these morphological changes, HSC gains new functions such as proliferation, migration, contractility, and protein synthesis\textsuperscript{15,54-59,68}. Activated HSC produce specialized enzymes such as matrix metalloproteases (MMP) that can destroy the normal pericellular ECM before producing fibrous tissue remodeling. Therefore, HSC’s have a key role in the control of liver fibrosis synthesis and destruction\textsuperscript{60,61}.

### 3.7 Initiation of Stellate Cell Activation

The earliest changes in stellate cells are likely to result from paracrine stimulation by all the neighboring cell types; including sinusoidal endothelium, Kupffer cells, hepatocytes, and platelets. Factors that trigger the initial step are called initiators\textsuperscript{1}.

Early injury to the endothelial cells stimulates the production of cellular fibronectin, which has an activating effect on stellate cells\textsuperscript{16}. Endothelial cells are also likely to participate in the conversion of TGF-β from a latent to very potent active fibrogenic form\textsuperscript{1}.

Kupffer cell infiltration and activation also play a prominent role. The influx of Kupffer cells coincides with the appearance of markers of stellate cell activation. Kupffer cells can stimulate matrix synthesis, cell proliferation, and release of retinoids by stellate cells through the actions of cytokines (especially TGF-β1) and reactive oxygen intermediaries/lipid peroxides\textsuperscript{1}.

Due to the critical role of the Kupffer cells in the mediation of liver injury and fibrogenesis, inhibiting Kupffer cell activity has been proposed as a means to reduce liver
injury. In the study as quoted in Prosser CC, Ogushi et al., modulated Kupffer cell function by administering double stranded antisense ODN targeting NF-κB, the p65 subunit. The mice were first sensitized with intraperitoneal injection of heat killed Propionibacterium acnes. Phosphorothionate modified antisense ODN was delivered with hemagglutinating virus of Japan (HVJ)-liposomal complexes via portal vein injection on day 4 and 7 after P. acnes priming challenge. The HVJ-liposome-mediated antisense ODN against NF-κB significantly improved animal survival, and the mortality was decreased to 15% in mice treated with NF-κB decoy ODN as compared to 90% in control mice that died within 24 hours. The treatment with decoy antisense ODN against NF-κB also suppressed the production of proinflammatory cytokines (IL-1β, TNF-α, IL-18, and IL-12) by Kupffer cells and decreased mRNA expression of IFN-γ and Fas-L. Less infiltration of inflammatory cells and lesser destruction of sinusoidal architecture were documented by histopathology in the animals treated with antisense ODN against NF-κB.

As mentioned by Friedman SL, in the initiation of stellate cell activation; the early stimulation of stellate cells by lipid peroxides in vivo may be important in many forms of liver fibrosis; particularly hepatitis C, nonalcoholic steatohepatitis (NASH), and iron overload. Because antioxidant levels are typically depleted in cirrhotic liver, as fibrosis advances, their loss may further amplify the injurious effects of lipid peroxides. Hepatocytes are the most abundant cells in the liver, are a potent source of fibrogenic lipid peroxides. A correlation has been noted in situ between the presence of aldehyde adducts and collagen gene expression by stellate cells, and peroxides stimulate collagen synthesis by cultured stellate cells. Steatosis in NASH and hepatitis C correlates with increased stellate cell activation and fibrogenesis, possibly because fat represents an enhanced source of lipid peroxides. In culture, activation of stellate cells is provoked by the generation of free radicals and is blocked by antioxidants. This activation may involve the transcription factors c-Myb and nuclear factor κB because antibodies to these factors disrupt activation. These and other key transcriptional events are an important early signs in the activation cascade.

Recently, peroxisome proliferator-activated receptors (PPAR’s) particularly PPAR-γ have been identified in stellate cells, and their expression increases with
activation \(^{79,80}\). Ligands for this newly identified nuclear receptor family down-regulate stellate cell activation \(^{1,80}\).

Platelets are an important paracrine stimulus, are present within the injured liver, and are a potent source of growth factors. Potentially important platelet mediators include PDGF, TGF-\(\beta_1\), and epidermal growth factor \(^1\).

### 3.8 Perpetuation of Stellate Cell Activation

According to Friedman SL \(^1\), the perpetuation of stellate cell activation involves at least seven changes in cell behaviour: proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, retinoid loss, white blood cell chemoattractant and cytokine release. Either directly or indirectly, the net effect of these changes is to increase the accumulation of extracellular matrix. For example, proliferation and chemotaxis lead to increased numbers of collagen producing cells, but matrix production per cell is also increased. Cytokine release by the stellate cells can amplify the inflammatory and fibrogenic tissue responses. Matrix proteases may hasten the replacement of normal matrix with matrix typical of the wound “scar” \(^1\).

#### i) Proliferation

An increase in stellate cell number has been well documented after both experimental and human liver injury \(^{81,82,83}\). Indeed, proliferation is an important component of the activation cascade because it amplifies the stellate cell–mediated response to injury. A number of mitogens appear to be important in stimulating stellate cell proliferation. These include PDGF \(^{56}\), epidermal growth factor \(^{84}\), fibroblast growth factor \(^{85}\), endothelin-1 \(^{86}\), insulin-like growth factor \(^{87}\), thrombin \(^{88}\), and transforming growth factor alpha (TGF-\(\alpha\)) \(^{85,89}\).

PDGF is the most potent stellate cell mitogen identified. The induction of PDGF receptors early in stellate cell activation increases responsiveness to this mitogen \(^{75,90}\). Downstream pathways of PDGF signaling have been carefully characterized in stellate cells \(^91\). In addition to proliferation, PDGF stimulates Na\(^+\)-H\(^+\) exchange, thereby providing a potential site for therapeutic intervention through the blockage of ion transport \(^{92,93}\).
Thus, neutralizing PDGF activity, by either ligand antagonists or receptor blockade, is a potentially important therapeutic approach.

In animal models of liver fibrosis, stellate cell proliferation is important in liver fibrosis, and it has been demonstrated that during spontaneous recovery of experimental liver fibrosis, stellate cell apoptosis (programmed cell death) was prominent. These data suggest that apoptosis of activated stellate cells may play a role in the resolution of fibrosis, and that a balance between cell proliferation and death is important in determining the dynamics of the total overall stellate-cell population in the liver.

ii) Chemotaxis

Stellate cells migrate towards cytokine chemoattractants. Such migration is also characteristic of wound infiltrating mesenchymal cells in other tissues. Chemotaxis of stellate cells explains in part why stellate cells align within inflammatory septa in vivo.

iii) Fibrogenesis

One of the earliest described and most prominent effects of stellate cell activation is increased extracellular matrix production. The available evidence now indicates that the overall increase in extracellular matrix deposition typical of cirrhosis can be largely ascribed to excess production by stellate cells.

Increased matrix production is the most direct way in which activated stellate cells generate hepatic fibrosis. Among the components of the hepatic scar, collagen type I is the best studied; and numerous reports describe the regulation of the collagen I gene in stellate cells.

The most potent stimulus to the collagen I production is TGF-β, which is derived from both paracrine and autocrine sources. TGF-β also stimulates the production of other matrix components, including cellular fibronectin, and proteoglycans. Transforming growth factor beta-1 seems to act by directly (and, to a lesser extent, indirectly) stimulating extracellular matrix production in stellate cells. Transforming growth factor beta-1 can be produced in a paracrine manner from Kupffer's cells or by stellate cells themselves, and thus can be an important autocrine factor.
When expression of TGF-β1 is blocked during experimental liver injury, fibrosis is reduced\(^\text{105}\).

According to Friedman SL\(^1\), TGF-β\(_1\) stimulates collagen in stellate cells through hydrogen peroxide- and C/EBP-β-dependent mechanism\(^\text{106}\). Stellate cell responsiveness to TGF-β\(_1\) is increased during activation by enhanced ligand binding to its cognate receptors\(^\text{107}\). Signals downstream of TGF-β include a family of bifunctional molecules known as SMAD’s, on which many extracellular and intracellular signals converge to fine tune and further enhance the effects of TGF-β during fibrogenesis downstream of its receptors\(^\text{91}\). The response of SMAD’s in stellate cells differs between acute and chronic injury to favour matrix production in chronic injury\(^\text{108}\). The increased expression of TGF-β in patients with chronic hepatitis C underscores the potential importance of this cytokine in chronic liver disease\(^\text{109}\).

Lipid peroxidation products are emerging as important stimuli to the production of extracellular matrix\(^6\). Their effects may be amplified by loss of the antioxidant capacity of stellate cells as they become activated\(^\text{110}\). These important insights have stimulated efforts to use antioxidants as therapy for hepatic fibrosis\(^1\).

Other cytokines and growth factors, many of which stimulate stellate cell proliferation, are important in the fibrogenic cascade. Platelet-derived growth factor (PDGF),\(^\text{56,111}\) monocyte chemotactic factor (MCP-1),\(^\text{59}\) hepatocyte growth factor,\(^\text{112}\) insulin-like growth factors (IGF-1 and 2),\(^\text{87}\) and interleukin-6\(^\text{113}\) are produced by stellate cells and seem to serve as autocrine stimulators of stellate-cell proliferation, in turn indirectly augmenting fibrogenesis.

Recent evidence also indicates that peptides such as endothelin-1, a vasoactive peptide that has pleiotropic biologic effects, are important in fibrogenesis. Abundant evidence in other wounding models indicates that this peptide stimulates extracellular matrix synthesis\(^\text{114,115,116}\). Indeed, endothelin-1 is overproduced in the cirrhotic liver\(^\text{117,86}\) and stimulates hepatic fibrogenesis\(^\text{118}\). Other biologically active peptides that are also important in fibrogenesis include angiotensin II, and adrenomedullin\(^\text{119,120}\). It is likely that other, as yet unidentified, biologically active peptides are important in mediating the hepatic fibrogenic cascade; some examples are other compounds, which are not
catalogued easily as peptides or cytokines (e.g., adiponectin, leptin, and prostanoids)\textsuperscript{121,122,123}.

Finally, although cytokines are important components of the fibrogenic response to injury, it is clear that stimuli underlying stellate cell fibrogenesis extend beyond cytokines such as TGF-β and include the matrix itself, which clearly modulates the activation state and thus the production of extracellular matrix by stellate cells. For example, culture of stellate cells on a basement membrane mimicking the normal basement membrane inhibits stellate cell activation and matrix synthesis,\textsuperscript{124} whereas culture of stellate cells on the EDA isoform of fibronectin leads to increased activation of stellate cells synthesis of matrix and smooth muscle proteins\textsuperscript{16}. A further example of the importance of the extracellular matrix comes from work that demonstrated that stellate cells which are exposed to an abnormal basement membrane (type I collagen) exhibited marked activation of MMP-2, which in turn would be predicted to degrade normal basement membrane extracellular matrix further\textsuperscript{125}. Finally, type I collagen promoted activation of hepatic stellate cells through discoidin domain tyrosine kinase receptor 2 (DDR2) signaling, which increased the expression of active MMP-2 and led to enhanced proliferation and invasion\textsuperscript{126}. Thus abnormal matrix in the injured hepatic environment seems to have critical consequences for cellular function and implies that the interruption of certain cell-matrix interactions could be therapeutically beneficial\textsuperscript{2}.

iv) Contractility

The contractility of stellate cells may be a major determinant of early and late increases in portal resistance during liver fibrosis. And this may be important in the pathophysiology of portal hypertension\textsuperscript{2}. Activated stellate cells impede blood flow both by constricting individual sinusoids and by contracting the cirrhotic liver. The collagen bands typical of end stage cirrhosis contain large numbers of activated stellate cell\textsuperscript{127}. The acquisition of a contractile phenotype during stellate cell activation has been documented both in culture and in vivo and is mediated in part by integrin receptors that interact with the extracellular matrix\textsuperscript{91}.  

\textsuperscript{120}
As stellate cells become contractile, their expression of the cytoskeletal protein α-smooth muscle actin and smooth muscle myosin is increased \cite{128,129}. This observation led to work demonstrating that stellate cells are contractile, \cite{130,131,132} a feature common to myofibroblasts in general \cite{24,133,134}.

Stellate cell contraction seems to be induced by a number of compounds, including endothelin-1 \cite{132}, prostanoids \cite{130}, substance P \cite{135}, angiotensin II \cite{131}, and arginine vasopressin \cite{119}.

The major contractile stimulus to stellate cells is endothelin 1; its receptors are expressed on both quiescent and activated stellate cells, but their subunit composition may vary \cite{136}. It is also postulated that, with activation, receptor expression doesn’t increase, unlike that of PDGF receptors, but a shift in the type of endothelin receptor that predominates is combined with increased sensitivity to autocrine endothelin 1 \cite{127}. Increased endothelin levels result from increased endothelin-converting enzyme activity that in turn is a result of stabilization of the endothelin-converting enzyme mRNA \cite{127}. The contractility of stellate cells in response to endothelin1 has also been observed in vivo \cite{137}. Other, less potent contractile stimuli have been identified \cite{127}.

Locally produced vasodilator substances may counteract the constrictive effects of endothelin1 \cite{127}. Nitric oxide, which is also produced by the stellate cells \cite{138}, or from the sinusoidal endothelium \cite{139}, is a well characterized endogenous antagonist to endothelin (Fig. 7). During acute endotoxemia, stellate cell production of nitric oxide is increased. Studies in vivo suggest that carbon monoxide \cite{140} and adrenomedullin \cite{141} also mediate sinusoidal relaxation through their effects on stellate cells \cite{1,2}.

According to Rockey DC \cite{2}, the clinical significance of increased stellate cell contractility during injury in vivo is a subject active investigation. Available data suggest that stellate cells controls sinusoidal blood flow by perisinusoidal constriction, analogous to the way that tissue pericytes control blood flow in systemic capillary structures \cite{142}. Because stellate cell contractility is greatest after stellate cell activation, and because endothelin-1 is overproduced in the injured liver, enhanced contractility after activation seems to contribute to elevated intrahepatic resistance to blood flow \cite{143,144}. Contraction of stellate cells residing within bands of extracellular matrix is likely to lead to the whole-organ contraction characteristic of end-stage liver disease \cite{145}.
If smooth muscle actin is required for contraction, then its inactivation may represent a target for treating portal hypertension.1

v) Matrix Degradation

Quantitative and qualitative changes in matrix protease activity play an important role in ECM remodeling that accompany fibrosing liver injury. Stellate cells express virtually all the key components required for pathologic matrix degradation and therefore play a key role not only in matrix production but also in matrix degradation.1 Discussed in detail later.

vi) Retinoid Loss

As stellate cells become activated, they lose their characteristic perinuclear retinoid (Vitamin A) droplets and acquire a more fibroblastic appearance. In culture, retinoid is stored as retinyl esters; however as stellate cells become activated, the retinoid release outside the cell is retinol, and intracellular hydrolysis of esters probably takes place before export.62 However, it is generally not known whether retinoid loss is required for stellate cells to become activated, and which retinoids may accelerate or prevent activation in vivo. For instance, one retinoic acid analogue, 9-cis-retinoic acid, stimulates hepatic fibrosis in rats by increasing the activation of latent TGF-β1.146

Several nuclear retinoid receptors have been identified in stellate cells,147 molecules that bind intracellular retinoid ligands and regulate gene expression, but it is uncertain whether they play a regulatory role in fibrogenesis. The question has important clinical implications because efforts are being made to use retinoids therapeutically.

vii) White Blood Cell Chemoattractant and Cytokine Release

Increased production or activity of cytokines may be critical for both autocrine and paracrine perpetuation of stellate cell activation. Direct effects on stellate cell matrix production and contractility have been attributed to autocrine TGF-β and endothelin1, respectively.1 Another important example is PDGF, which is produced by stellate cells
and binds to stellate cell PDGF receptors, it has prominent effects on the stimulation of stellate cell proliferation. Discussed in detail later.

In addition to TGF-β1, and PDGF, stellate cells are the source of various other cytokines and small peptides. For example, stellate cells secrete macrophage colony-stimulating factor (M-CSF), which may contribute to recruitment and activation of resident or infiltrating cells in the injured liver, and monocyte chemotactic protein-1. In addition to this mononuclear cell chemoattractant, stellate cells produce neutrophil chemoattractants, which may contribute to the neutrophil accumulation characteristic of alcoholic liver disease.

Recent studies have further demonstrated that stellate cells produce connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF), the latter of which may prove to be mitogenic, and many others, which often have effects on stellate cells themselves.

Finally, stellate cells also seem to produce cytokines that dampen the fibrogenic response, suggesting that stellate cells themselves could play a role in inhibiting fibrogenesis. For example stellate cells produce potent immunomodulatory cytokine interleukin (IL)-10. This cytokine has profound inhibitory actions on macrophages, cells that produce several cytokines themselves that modify the wound response. Stellate cells also produce hepatocyte growth factor (HGF), which has recently been shown to inhibit hepatic fibrogenesis, and has the potential to be mitogenic.

### 3.9 Cytokines & Small Peptides involved in Hepatic Fibrogenesis & Stellate Cell Activation

In addition to the hepatic stellate cells, Kupffer cells and sinusoidal endothelial cells; various cytokines play an important role in the hepatic fibrogenesis. Several general points merit emphasis:

i) Multiple cytokines are involved in the fibrogenic process, many of which are unknown.
ii) The effects of cytokines are diverse and complex; and the functions of even known cytokines are not fully defined yet.²

1) For example, PDGF stimulates stellate cell proliferation and also stimulates stellate cell motility, a function that is probably important in the fibrogenic response.¹⁵⁶

2) TGF-β1, although a potent profibrogenic cytokine, is also antiproliferative. Thus, the net effect of TGF-β1 on fibrogenesis may be mixed, but more fibrogenic.

3) Interferon-γ itself appears to have direct antifibrotic effects on stellate cells and may also inhibit interleukin-4 (IL-4) an apparently profibrotic cytokine. In turn, IL-4 may also induce TGF-β; which is a potent profibrogenic cytokine. Thus, it can be readily understood that interferon-γ may have multiple downstream effects.

iii) Although the effects of certain cytokines are well characterized in controlled experimental conditions in vitro, their effects in the complex extracellular environment in vivo; that includes interactions with other cytokines and extracellular matrix, may be more difficult to predict.

iv) This complexity raises questions about which cytokines represent the most reasonable therapeutic targets.

v) Hepatic fibrogenesis is a dynamic phenomenon. Both the cellular source and the target of cytokines in the liver fibrogenic response varies. This variability has important implications for therapeutic measures directed at specific cellular sources.

There are various cytokines involved in hepatic fibrogenesis. Stellate cells themselves are an important source of several cytokines and biologically active peptides which have effects not only on themselves (autocrine effect), (e.g., TGF-β1 in fibrogenesis, PDGF and others in proliferation, endothelin-1 in contractility), but also on other cells (paracrine effect) in the hepatic-wounding environment (for e.g., Interleukin-10, colony stimulating factor, and monocyte chemotactic peptide-1.²⁵⁹
The multiple cytokines that are produced during the hepatic fibrogenic response have diverse effects which include; but are not limited to: stimulation of inflammation, cell growth, and fibrogenesis (as well as their inhibition). Cytokines can be divided into those that are primarily fibrogenic, that stimulate growth, that are immunomodulatory, and those that are chemotactic.2

3.10 Tyrosine Kinase Receptor Ligands

Two of the most prominent cytokines produced in the injured liver are the tyrosine kinase receptor ligands TGF-β and PDGF. As described previously, these cytokines directly mediate stellate cell fibrogenesis and proliferation, respectively.

i) Transforming Growth Factor-β (TGF-β)

TGF-β is the most potent cytokine for enhancing hepatic fibrogenesis; and has received the most attention. It is a tyrosine kinase receptor ligand. It suppresses hepatocyte proliferation, stimulates the activation of HSC, promotes ECM production, and mediates hepatocyte apoptosis101,161,162.

Among the most convincing pieces of evidence indicating the involvement of TGF-β1 in hepatic fibrogenesis is the profibrotic effect of transgenically expressed TGF-β1 in hepatocytes99; further work which blocked TGF-β production in the liver confirmed its critical role105. The effect of TGF-β1 seems to be largely through direct and indirect stimulation of extracellular matrix production in stellate cells101,162. Sources of TGF-β1 are paracrine from Kupffer cells102 and autocrine103,104 from stellate cells. Increased binding of TGF-β1 has also been documented during stellate cell activation in vivo and in culture,107 and increased activation of latent TGF-β1 may also contribute to a net increase in TGF-β activity163.

Soluble TGF-β decoy receptors or adenoviral constructs that block TGF-β signaling have been developed that show antifibrotic efficacy in vitro and in vivo105,161,164,165.

As mentioned by Prosser CC,3 Smad3 (a transcriptional factor in TGF-β receptor downstream signal transduction) knock out mice did not develop hepatic fibrosis166;
while transgenic mice overexpressing TGF-β1 developed hepatic fibrosis faster than wild type mice, and the fibrosis regressed slower after the withdrawal of the fibrogenic agent.

For example, Qi et al.\textsuperscript{105} evaluated adenoviral expression of truncated TGF-β receptor type II to abolish TGF-β signaling in liver fibrosis mediated by dimethylnitrosamine. Sprague-Dawley rats were given a single infusion of adenoviral vectors (AdCATβ-TR) encoding the TGF-β receptor II gene (TGF-βRII) via portal vein injection. A greater than 20 fold increase in truncated receptor mRNA expression with the adenoviral gene delivery was detected in animals after the TGF-β receptor type II gene delivery with adenoviral vectors. The gene delivery improved the animal mortality, decreased liver hydroxyproline content by 3.4 fold, hyaluronate levels by nearly 20 fold, AST by 100 fold, and ALT by 63 fold. Less hepatocyte injury and fibrosis with a decreased infiltration of monocytes/macrophages and reduced semiquantitative score of fibrosis in histopathology were noted; which was consistent with decreased gene expression of collagen type I, fibronectin, smooth muscle α-actin (SMA), and TGF-β1 in the treated animals\textsuperscript{3}.

With the importance of TGF-β1 for extracellular matrix production firmly established, efforts are under way to develop therapies which neutralize this cytokine. Its inhibition, therefore appears attractive\textsuperscript{9,161,169}. It appears that an approach targeting activated HSC and MF is necessary, since TGF-β receptors are expressed on most cell types and systemic inhibition that reaches sufficient levels to block hepatic fibrogenesis may trigger autoimmune diseases and cellular de-differentiation\textsuperscript{155}.

\textbf{ii) Platelet Derived Growth Factor (PDGF)}

Another important cytokine of the tyrosine kinase receptor ligand type is the PDGF. It is the most potent mitogen for HSC with effects in growth stimulation, chemotaxis, and intracellular signaling. PDGF expression is upregulated in hepatic injury, as are PDGF receptors in activated HSC\textsuperscript{3,170}.

Stellate cells express PDGF A- and B-chain mRNA and release bioactive PDGF, indicating that PDGF serves as an important autocrine factor during liver injury\textsuperscript{111}. Although regulation of PDGF production in stellate cells is complex (and intricately
related to other events in the wounding milieu)\textsuperscript{171,172}, this regulation is critical in the fibrogenic cascade because PDGF seems to be the most potent stimulus of stellate cell proliferation\textsuperscript{85}.

According to Prosser CC,\textsuperscript{3} the tyrosine kinase activity of PDGF receptors signals through PI-3K, Ras, Raf-1, and ERK, leading to nuclear translocation and activation of nuclear transcription factors\textsuperscript{173}. Interrupting PDGF signal effects with specific agents inhibiting tyrosine phosphorylation, or interrupting down stream signal transduction pathways attenuates hepatic fibrosis by preventing proliferation and chemotaxis of HSC in vitro and in vivo\textsuperscript{173}. Selectively targeting the PDGF receptor by specific antibodies or agents has been considered a valuable strategy to block the progression of hepatic fibrogenesis\textsuperscript{22,174}.

### 3.11 Immunomodulatory Cytokines

Immune mediated damage is linked to fibrogenesis, but the character of the immune response determines fibrosis progression. Contrary to the general thinking, fibrosis appears not to be the logical consequence of significant macroscopic inflammation as reflected by the mononuclear infiltrate; but rather by the associated intrinsic and extrinsic immunosuppression. Here the cytokines play an important role\textsuperscript{155}.

The immunomodulatory cytokines include tumor necrosis factor-alpha (TNF-\(\alpha\)), the interleukins, and the interferons (-\(\alpha\), -\(\beta\), and -\(\gamma\)). Immunomodulatory cytokines are produced by lymphocytes, natural-killer (NK) cells and macrophages and typically can be divided into proinflammatory Th1 cytokines (interferon-\(\gamma\), interleukins 2, 3, and 12, and TNF-\(\alpha\)) and anti-inflammatory, profibrogenic Th2 cytokines (interleukins 4, 5, 9, 10, and 13)\textsuperscript{2}.

They can be subdivided into fibrogenic, antifibrogenic, and neutral group. The later has no effect on fibrogenic effector cells, i.e., the HSC and the myofibroblasts (MF)\textsuperscript{155}.

As a general rule, certain Th1 cytokines that stimulate cellular immune responses, (proinflammatory) rather trigger matrix dissolution, i.e., fibrolysis. Whereas Th2
cytokines, which stimulate the humoral immune response and can suppress Th1 T cells promote fibrogenesis.\textsuperscript{155}

Evidence comes from patients coinfected with both HIV(Th2) and HCV (Th1 in acute, Th2 in chronic infection) who progress more quickly to cirrhosis than patients infected with HCV alone.\textsuperscript{175} Similarly accelerated fibrosis progression is found in HCV patients coinfected with Schistosoma mansoni which triggers a Th2 cytokine profile.\textsuperscript{176}

\textbf{i) Interleukin-10}

IL-10 seems to have effects similar to those of interferon-\(\gamma\) in the wounding milieu. Activated stellate cells produce this cytokine, which has potent inhibitory actions on macrophages.\textsuperscript{152,153} Macrophages are cells that also produce a several cytokines that modify the wounding response. In culture, IL-10 was found to be antifibrogenic toward stellate cells. The potential importance of this cytokine is underlined by the finding that IL-10 knock-out mice exhibited significantly more fibrosis and higher hepatic TNF levels than wild-type controls.\textsuperscript{177} This finding suggests that IL-10 synthesized during fibrogenesis may modulate Kupffer's cells and serve as an antifibrotic cytokine, much like interferon-\(\gamma\).\textsuperscript{178}

\textbf{ii) Tumor Necrosis Factor-\(\alpha\) (TNF-\(\alpha\))}

According to Prosser CC,\textsuperscript{3} TNF-\(\alpha\) is a key cytokine involved in many forms of liver injury, and may play a crucial role in HSC activation and hepatocyte regeneration.\textsuperscript{151,179} The major cell type for TNF-\(\alpha\) production in the liver is Kupffer cells which release TNF-\(\alpha\) when activated by factors released by damaged hepatocytes, and by Reactive Oxygen Species, ROS. TNF-\(\alpha\) participates in the second phase of hepatocellular damage via apoptosis and TRAIL receptor activation in alcoholic or other hepatotoxic induced liver injury.\textsuperscript{180} It acts on HSC, and may contribute to the activation process.\textsuperscript{181} Accordingly, reducing TNF-\(\alpha\) production, or blocking its action will significantly minimize liver injury caused by alcohol toxicity, acetaminophen overdose, or ischemia/reperfusion-associated liver injury in animal models.\textsuperscript{180,182}
3.12 INTERFERONS (The Immunomodulatory Cytokines)

The interferons are unique biological compounds with major immunomodulatory, anti-viral and anti-fibrogenic effects. Three major isoforms of interferon exist (α, β, and γ) \(^{183}\). Each of these isoforms is unique in protein structure and in biologic actions \(^ {178}\).

There are multiple interferon-α subtypes but only single interferon-β and interferon γ-species. Interferon-α and interferon-β are more closely related to each other, structurally (indeed, they each bind to the same receptor) and functionally, than they are to interferon-γ. Interferon-α is stimulated by viral infection, whereas interferon-γ is stimulated by mitogenic or antigenic stimulation of T lymphocytes or NK cells \(^ {178}\).

The major interferon isoforms, α and γ, each bind to unique receptors. Interferon-α has much more potent antiviral effects than does interferon-γ. On the other hand interferon-γ is 100 to 10,000 times more potent as an immunomodulator than interferon-α \(^ {184}\). This observation has led to the belief that whereas interferon-α and -β are primarily antiviral agents that have some immunomodulatory effects, interferon-γ is primarily an immunomodulatory agent with some antiviral effects \(^ {178}\).

i) Interferon-α

It is well established that interferon-α is capable of eradicating hepatitis virus B and hepatitis virus C from the liver. Evidence clearly indicates that for hepatitis C this eradication is associated with a reduction in fibrosis \(^ {109,185-190}\). Studies in patients with chronic hepatitis C suggest that IFN-α therapy can prevent fibrosis progression, even in nonresponders to antiviral therapy \(^ {191-194}\). The effect was dependent on IFN-α dose duration and most pronounced in sustained responders. A study has found reversion of cirrhosis in 75 out of 153 patients \(^ {155,193}\).

Although some experimental evidence suggests that interferon-α has primary antifibrogenic effects \(^ {195,196}\) (which may play a role in reducing fibrogenesis in patients, independent of its effect on hepatitis C), these data are not been nearly as convincing as those for interferon-γ \(^ {178}\). This effect is linked at least, in part, to the activation of stat-1 signalling pathways. In cell culture, interferon-α (IFN-α) blocks activation, proliferation, and collagen synthesis of HSC and MF \(^ {197}\).
ii) Interferon-γ

According to Rockey DC \(^{178}\), interferon-γ is ineffective at eradicating hepatitis virus B or C, but extensive experimental evidence indicates that interferon-γ effectively inhibits fibrogenesis in the liver \(^{198-201}\) and in other organs \(^{202,203}\). The mechanism for the effect in the liver seems to be a global inhibition of stellate cell activation, \(^{157,159,198,200}\) although the mechanism by which activation is inhibited remains unclear. It may also inhibit interleukin-4 (IL-4) \(^{204}\), an apparently profibrotic cytokine \(^{159}\).

There has been little clinical interest in the use of interferon-γ in patients with hepatic fibrogenesis because the overexpression of interferon-γ in the liver leads to chronic hepatitis \(^{205}\) and because long-term side effects may result from its profound immunomodulatory effects \(^{178}\). A report in patients with pulmonary fibrogenesis, however, suggests that it may be feasible to use interferon-γ to treat patients with hepatic fibrogenesis \(^{206}\).

3.13 Chemotactic Cytokines / Chemokines

According to Rockey DC \(^{178}\), chemotactic cytokines or chemokines of the C-X-C family (interleukin-8, platelet factor [PF]-4) are neutrophil chemoattractants, whereas those of the C-C family (MCP-1, macrophage inflammatory protein [MIP]-1, and regulated on activation of normal T cell expressed and secreted [RANTES]) are chemotactic for mononuclear cells. Several of these cytokines, including colony-stimulating factor \(^{158}\) and monocyte chemotactic peptide-1 \(^{159}\), are secreted by stellate cells and probably contribute to recruitment of inflammatory cells after injury.

3.14 Peptides

i) Endothelins

As mentioned by Rockey DC \(^{178}\), the peptide that has received the most attention is endothelin \(^{207}\). This family of potent vasoconstrictors \(^{208}\) comprises three unique endothelin peptides, each consisting of 21 amino acids, which have been termed endothelin-1, endothelin-2 and endothelin-3 \(^{209}\). They bind to at least two heptahelical (G-protein–coupled) receptors, termed endothelin A (ET\(_A\)) and endothelin B (ET\(_B\)) receptors.
Endothelins are usually produced by endothelial cells and exert paracrine effects on adjacent smooth muscle cells. In this capacity, it is believed that their major role is the local control of vascular tone, including regulation of basal blood pressure. The endothelins induce vasoconstriction by stimulating ET, receptors on smooth muscle cells and to induce vasodilation by stimulating ET, receptors on endothelial cells. Although the vasoregulatory functions of the endothelins have been emphasized here, reports also emphasize regulation growth and other effects.

In liver, endothelins are produced by sinusoidal endothelial cells. There is substantial evidence that endothelin-1 is involved in regulating sinusoidal blood flow. Accumulating evidence indicates that endothelin biology extends far beyond vasoregulation. Indeed, a large body of work now indicates that the endothelins are involved in the wound-healing response. Available data further indicate that the source of endothelin is the injured tissue itself. In some systems, the cellular compartments responsible for increased production of endothelin have been identified. For example, in liver, the cellular source of endothelin in the liver shifts from the sinusoidal endothelial cell to the hepatic stellate cell after injury. Also, the total amount of endothelin in the liver is overproduced, and because stellate cells have many ET, and ET, receptors, endothelin-1 has multiple effects on stellate cells, apparently in an autocrine fashion. Endothelin-1 has potent effects on stellate cell contractility; as described earlier, this effect is probably involved in elevated intrahepatic resistance to blood flow. Finally, as in other systems, overproduced endothelin in the injured liver appears to have major effect on the fibrogenic response to injury.

The mechanism underlying increased endothelin-1 synthesis seems to be based on the regulation of endothelin converting enzyme-1, which converts precursor endothelin-1 to mature endothelin-1. Further, TGF-β seems to control endothelin-1 production, at least in part, through modulation of endothelin converting enzyme-1. This latter finding emphasizes the complex inter-relationships by which cytokines control fibrogenesis.
3.15 Reactive Oxygen Species and Free Radical Scavengers

Oxidative stress is an important mechanism in liver injury. ROS include superoxide anions, hydroxyl radicals, hydrogen peroxide, and hydroxyethyl radicals (HER). These are generated from a variety of insults, such as drugs/toxin metabolites, ischemia/reperfusion, and alcohol metabolism. ROS are involved in necrosis and apoptosis of hepatocytes, and contribute to HSC activation. Several major classes of free radical scavengers, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-P), as well as SOD mimics, were investigated in various forms of liver injury, and they afforded effective protection against the oxidative insults to the tissue.

3.16 Composition of Extracellular Matrix in Normal Liver & Hepatic Scar

The extracellular matrix refers to the array of macromolecules comprising the normal and fibrotic liver. Its components include several families of structural and nonstructural molecules: collagens, noncollagen glycoproteins, matrix-bound growth factors, glycosaminoglycans, proteoglycans, and matricellular proteins. As described in detail by Freidman SL, remarkable progress has been made in identifying new members of these families, and in understanding how these molecules interact. Matrix composition within different tissue regions is markedly heterogenous in respect to the various isoforms within each class of molecules, their stoichiometry, and their intermolecular interactions. Moreover, hybrid molecules have been identified that may contain, for example, both collagenous and proteoglycan domains. Additionally, a variety of new roles of matrix molecules are now recognized, including their role as transmembrane transducers of extracellular signals.

The most abundant proteins in ECM are collagens, a family of proteins containing 19 isotypes with a common trihelicoidal structure. Of the 20 types of collagens characterized thus far, 10 have been identified in liver. On a morphological basis, collagens can be divided into those forming fibrils (interstitial collagens) and those
included within the basement membranes. In the normal liver, so-called fibril-forming collagens (type I, III, V, and XI) are largely confined to the capsule, around large vessels, and in the portal triad. Only scattered fibrils containing types I and III collagen are found in the subendothelial space. Additionally, smaller amounts of other collagens, including types VI, XIV, and XVIII are found. Glycoproteins and matricellular proteins are also present, including subendothelial deposits of fibronectin, laminin, tenascin, and von Willebrand factor. The proteoglycans, which are primarily of heparan sulfate proteoglycans, include perlecan, and small amounts of decorin, biglycan, fibromodulin, aggrecan, glypican, syndecan, and lumican.

The matrix composition of fibrotic liver differs both quantitatively and qualitatively from that of normal liver; and these changes are similar regardless of the type of liver injury. The total collagen increases 3 to 10 fold. For instance, in the normal liver, ECM comprises less than 3% of area on a liver cut section. In fibrosis, quantitative increase (30%–40% in cirrhosis) and composition modification of ECM are observed, leading to liver architecture distortion and impairing of the exchanges between blood and liver cells. Important to note is: basement membranes are usually absent along the sinusoids but are deposited during the process of cirrhosis, termed “sinusoid capillarisation”.

Although, in the fibrotic liver, there is ECM modification, but the collagen is not “abnormal” in sequence or structure. Overall, the “interstitial matrix” typical of a healing wound is markedly increased and includes fibril forming collagens (types I, III, V), some non-fibrill forming collagens (types IV, and VI), several glycoproteins (cellular fibronectin, laminin, osteonectin, tenascin, and von Willebrand factor), and a large number of proteoglycans and glycosaminoglycans (perlecan, decorin, aggrecan, lumican, fibromodulin). In particular, a shift occurs from heparin sulfate-containing proteoglycans to those containing chondroitin and dermatan sulfates.
3.17 Biological Activity of Extracellular Matrix in Liver

i) Accumulation of Subendothelial Matrix

Although a prominent and highly visible effect of liver injury is the dramatic accumulation of extracellular matrix, it is important to understand that the extracellular matrix itself is highly dynamic and has profound effects on hepatic cellular function.

Normally the subendothelial space contains the components of a basement membrane, that, unlike true basement membrane, is not electron dense. These components include the following: laminin, type IV collagen, some types I, III, V, and VI collagen, the FACIT (fibril associated collagens with interrupted triple helices), collagen type XIV; and several heparin sulphate proteoglycans, including decorin, perlecan, syndecans 1 through 4, and glypican.

The basement membrane constituents within the subendothelial space may be essential to preserving the differentiated functions of hepatocytes, hepatic stellate cells, and subendothelial cells.

For example, studies in cultured cells have demonstrated that the maintenance of differentiated hepatocyte function requires contact with a matrix substratum similar to that found in the subendothelial space of normal liver. A hallmark of early liver injury is the replacement of this normal subendothelial matrix, which contains laminin, type IV collagen, and fibronectin, with one enriched in interstitial collagens types I and III. The high density matrix also activates stellate cells, and leads to a decrease in the fenestrations of sinusoidal epithelial cells, which may impair the transport of solutes from the sinusoid to the hepatocytes. Early accumulation of subendothelial matrix that leads to “capillarisation” of the subendothelial space of Disse is a key event, that may be more important than the overall increases in the matrix constituents.

This replacement may lead to deterioration of hepatocellular function and may alter the biology of other cells in the subendothelial space of Disse. Indeed, clinical hallmarks of chronic liver disease, such as impaired albumin and clotting factor synthesis,
almost certainly are in part the result of altered hepatocyte function caused by an altered microenvironment.

Recent studies have begun to elucidate the mechanisms underlying changes in the basement membrane extracellular matrix, and this understanding may ultimately lead to new therapies. An early component of the altered subendothelial matrix is the fibronectin isoform known as EDA (or cellular) fibronectin. This matrix protein is produced by sinusoidal endothelial cells very early in the injury response. Once synthesized, it is, in turn, capable of directly activating stellate cells, leading to enhanced stellate cell synthesis of matrix and smooth muscle proteins. A further example of the interaction between matrix and cells has been recently described in that stellate cells exposed to an abnormal basement membrane (type I collagen) exhibited marked activation of MMP-2, which, in turn, would be expected to cause further degradation of the normal basement membrane extracellular matrix further. Further type I collagen promoted activation of hepatic stellate cells through discoidin domain tyrosine kinase receptor 2 (DDR2) signaling, which increased the expression of active MMP-2 and led to enhanced proliferation and invasion.

Thus, abnormal matrix in the injured environment seems to have critical consequences for cellular function. Interrupting certain cell-matrix interactions could be therapeutically beneficial.

ii) Integrins

As discussed by Rockey DC, a major class of membrane receptors that mediate cell-matrix interactions are the family of proteins known as integrins. Altered cellular behavior induced by matrix alterations is typically mediated by these cell membrane receptors.

Integrins, are the best characterized type of extracellular matrix receptors. These are a large family of homologous membrane linker proteins. Integrins are noncovalent αβ heterodimers (made up of an alpha chain and a beta chain), that consist of a large extracellular domain, a membrane-spanning domain, and a cytoplasmic tail.

Integrins control many cellular functions, including gene expression, growth, and differentiation. A growing number of α and β subunits have been identified, with each
combination having a different cellular and ligand specificity. Integrins recognize a number of motifs on extracellular proteins, including the amino acids Arg-Gly-Asp (RGD) which mediate several important cellular functions. Integrin signaling across the plasma membrane allows communication between the extracellular matrix and cytoskeleton, in association with phosphorylation of several intracellular substrates. In addition to this signaling “outside in”, typical of most transmembrane receptors, integrins also signal “inside out”, meaning that integrin mediated cytoskeleton changes can lead to an altered conformation of extracellular matrix molecules-for example, fibronectin.

Several integrin and nonintegrin receptors have been identified in situ on hepatocytes and nonparenchymal cells. In liver, hepatic stellate cells express the integrin α1 β1, a collagen-binding integrin that mediates stellate cell adhesion to type I collagen and also stellate cell contraction of collagen I lattices. Stellate cells express a multitude of other integrins that are important in a variety of responses in the wound milieu.

Up-regulation of α6β1 and α5β1 receptors, both of which bind laminin, has been reported in experimental fibrosis. Studies have also reported the integrin phenotypes of isolated cell types from liver. In particular, stellate cells express integrin receptors for collagen and laminin, which may contribute to their activation in response to deposition of these matrix components during injury.

Antagonists directed against RGD sites are being widely investigated in many processes in which integrin binding is important (e.g., platelet adhesion and T-cell activation). Such antagonists may be involved in decreasing the severity of hepatic fibrosis through RGD-mediated or other pathways. For example, the RGD mimic, SF-6,5 markedly inhibited the progression of thioacetamide-induced liver cirrhosis. Treatment of stellate cells with soluble RGD peptides was also found to reduce the accumulation of type I collagen, whereas a control peptide had no such effect. Finally, it has been shown that the αvβ6 integrin binds and activates latent TGF-β1, indicating that integrins are involved in mediating cell matrix interactions and also in controlling the activity of other important components within the wounding environment.
ii) Other Cell Matrix Receptors

In addition to integrins, a growing number of other adhesion proteins and cell matrix receptors have been characterized, including \textit{cadherins} and \textit{selectins}, which mediate interactions between inflammatory cells and the endothelial wall \textsuperscript{248-250}. Up-regulation of a tyrosine kinase receptor, discoidin domain receptor 2 (DDR-2), has been identified during stellate cell activation; its signaling in response to fibrillar collagens leads to enhanced matrix metalloproteinase (MMP) expression and cell growth \textsuperscript{126}. DDR-2 is the only receptor tyrosine kinase with a ligand that is an extracellular matrix molecule rather than a peptide ligand, and its regulation may be a critical requirement for perpetuating liver fibrosis \textsuperscript{1}.

3.18 Remodeling/Degradation of Extracellular Matrix

The degradation of extracellular matrix represents a very important component of hepatic fibrosis due the following two reasons:

i) Early disruption of normal hepatic matrix by matrix proteases, hastens replacement of the normal matrix by scar matrix, which in turn has deleterious effect on cell function.

ii) In patients with chronic liver disease, and established fibrosis, the resorption of excess wound matrix, “therapeutic matrix degradation”, is urgently needed to arrest or reverse hepatic dysfunction and portal hypertension. Because fibrosis reflects a balance between matrix production and degradation, this balance must be shifted in favour of degradation for any antifibrotic therapy to succeed \textsuperscript{1}.

Although the net result of fibrogenesis is typically a progressive accumulation of excess extracellular matrix, fibrogenesis clearly involves a dynamic interplay between matrix synthesis and degradation. Under normal circumstances, a temporal and spatial balance between matrix synthesis and degradation exists, and restoring this balance leads to physical and functional restitution of the organ. In contrast, under abnormal circumstances, the disrupted balance leads to scar formation. The molecular processes
that control the balance between normal and abnormal synthesis and the degradation of extracellular matrix are only now becoming understood.

Significant progress has been made in elucidating the fundamental mechanisms of matrix remodeling and how these apply to hepatic fibrosis.

An enlarging family of Matrix Metalloproteinases (MMP’s), also known as Matrixins has been identified. These are calcium dependent enzymes that specifically degrade collagens and non-collagen substrates. They can also be inhibited by binding to specific inhibitors known as Tissue Inhibitors of Metalloproteinases (TIMP’s).

As suggested previously, the remodeling process is mediated by key cellular elements, among which are stellate cells. Stellate cells seem to accelerate the replacement of the normal subendothelial matrix by one rich in abnormal matrix constituents through secretion MMPs, such as MMP-1, type IV collagenase (MMP-2), or gelatinase, and through modulation of TIMPs.

i) Matrix Metalloproteinases (MMP’s)

Broadly, they fall into five categories based on substrate specificity:

i) Interstitial Collagenases
(MMP’s-1, -8, and -13)
The collagenases (interstitial collagenases that include MMP-1, -8, and -13 and the collagenase/gelatinase family) degrade a wide variety of collagens.

ii) Gelatinases
Gelatinase A (MMP-2), Gelatinase B (MMP-9), and fibroblast activation protein.
These degrade denatured collagens or gelatins and also digest native type IV collagen, an important component of the normal basement membrane.

iii) Stromelysins (MMP’s -3, -7, -10, and -11)
The stromelysins (-1, -2, and -3) degrade fibronectin, proteoglycans, laminin, and a variety of other matrix proteins.

iv) Membrane type (MMP’s -14, -15, -16, -17, -24, and -25), and
v) Metalloelastase (MMP-12)
MMP’s are regulated at many levels to restrict their activity to discreet regions within the pericellular milieu. The extracellular activity of the MMPs is tightly regulated by the interaction of activation pathways and specific inhibitors. The most prominent activation systems are the uroplasminogen activator (uPA) and tissue plasminogen activator (tPA) systems.\(^2\)

Inactive MMP’s can be activated through proteolytic cleavage by either membrane-type matrix metalloproteinase-1 (MT1-MMP), or plasmin\(^1\).

They can also be inhibited by binding to specific inhibitors known as Tissue Inhibitors of Metalloproteinases (TIMP’s)\(^1,2\).

The stoichiometry and molecular basis of these interactions has been greatly clarified. For example, membrane type matrix MMP-1 and TIMP-2 form a ternary complex with MMP-2, possibly including αvβ3-integrin, which is essential for optimal MMP-2 activity.\(^251\) Plasmin activity is controlled by its activating enzyme uroplasminogen activator (u-PA) and a specific inhibitor, plasminogen activator inhibitor 1 (PAI-1), and can be stimulated by active transforming growth factor β\(_1\) (TGF-β\(_1\)). Thus net collagenase activity reflects the relative amounts of activated MMP’s and their inhibitors, especially TIMP’s\(^1\).

In addition to TIMP’s other protease inhibitors may effect net degradative activity, including α2-macroglobulin\(^1\).

ii) Disruption of Normal Subendothelial Matrix - Initiation of fibrogenesis

Several matrix-degrading enzymes are produced by stellate cells during fibrogenesis.\(^252,258\) In the liver, “pathologic” matrix degradation is the early disruption of the normal subendothelial matrix, which occurs through the actions of at least four enzymes: MMP-2 (gelatinase A or 72-kd type IV collagenase) and MMP-9 (gelatinase B or 92-kd type IV collagenase), which both degrade type IV collagen; MT1-MMP, which activates latent MMP-2; and stromelysin 1, which degrades proteoglycans and glycoproteins and also activates latent collagenases. Stellate cells are a key source of MMP-2 \(^252,259\) and stromelysin \(^260\).
Stellate cells express the mRNA for the 72 kda type IV collagenase/gelatinase (MMP-2) and secrete this enzyme, particularly after activation. Activation of latent MMP-2 may require interaction with hepatocytes. Markedly increased expression of MMP-2 is characteristic of cirrhosis.

HCV envelope E2 glycoprotein binds to stellate cells, and induces an increased expression of MMP-2. This leads to increased degradation of the normal hepatic extracellular matrix, which in turn, facilitates stellate cell activation and fibrogenesis. Additionally, activation of DDR2 in stellate cells leads to increased expression of active MMP-2.

MMP-9 is secreted locally by Kupffer cells. Stimulation with IL-1α causes robust induction of pro-MMP-9 (the pre-cursor of MMP-9) in stellate cells and induces conversion of pro-MMP-9 to the active form when the cells are exposed to type I collagen.

Disruption of the normal liver matrix is also a requirement for tumor invasion and desmplasia.

Because the enzyme/s exhibits degradative activity against basement-membrane collagen, their release by activated hepatic stellate cells in the space of Disse disrupts the normal subendothelial liver matrix. Enhanced production of abnormal interstitial collagens (i.e., types I and III) subsequently leads to an abnormal basement membrane, which, in turn, disrupts hepatocellular function and may lead to further stellate cell activation. The net effect of this activity seems to be to accelerate the replacement of the normal subendothelial matrix by one rich in abnormal scar constituents.

Urokinase plasminogen activator generates plasmin and is inhibited by plasminogen-activator inhibitor (PAI)-1; uPA is localized to the cell surface by being bound to a specific receptor. Plasmin degrades extracellular matrix both directly and by activation of MMPs. After stellate cell activation, net uPA activity was increased, elucidating a mechanism for increased MMP activation.
ii) Progression of fibrogenesis & fibrosis

More importantly, progressive fibrosis is associated with marked increases in TIMP-1\textsuperscript{94,267}, and TIMP-2 \textsuperscript{268}, which lead to a net decrease in protease activity and therefore an increase in unopposed matrix accumulation. Stellate cells are the major source of these inhibitors\textsuperscript{269}. Sustained TIMP-1 expression is a key factor in progressive fibrosis, and its diminution is an important prerequisite for the reversal of fibrosis\textsuperscript{1}.

Moreover, TIMP-1 activity is increased in the fibrotic liver and with stellate cell activation, leading to an imbalanced expression of TIMPs relative to interstitial collagenase. This imbalance may promote the deposition of interstitial collagens in liver fibrosis\textsuperscript{254}. An enhanced ability to convert precursor forms of these enzymes to active forms after activation may help accelerate fibrosis\textsuperscript{253}.

From a practical standpoint, failure to degrade the increased interstitial or scar matrix is a major determinant of progressive fibrosis. MMP-1 is the main protease that degrades type I collagen, the principal collagen in the fibrotic liver; however, sources of this enzyme are not clearly established as those of the type IV collagenases. Stellate cells express MMP-1 messenger RNA (mRNA), but little enzyme can be detected\textsuperscript{259}.

Unique mechanisms of TIMP-1 regulation in stellate cells\textsuperscript{270}, offer the potential for the selective inhibition of TIMP-1 expression to accelerate the resorption of scar matrix\textsuperscript{1}.

3.19 Specificity of Liver Fibrosis in Chronic Hepatitis C

Because of the lack of HCV experimental models, specificities regarding cellular and molecular mechanisms involved in relation between HCV and fibrosis have not been explored in great detail at the molecular level, and the role of viral proteins in hepatic fibrosis deserve further study. Although several co-factors related to the host have been associated with a faster rate of fibrosis progression in hepatitis C, understanding of viral specific factors and their role in the evolution of fibrosis require further understanding\textsuperscript{193}; nevertheless, the representative work is submitted:
3.20 Are There Fibrogenic HCV Proteins?

A major impediment to the development of specific antiviral drugs has been the lack of a highly replicative and low cost in vitro or in vivo model of HCV infection. Several studies with HCV-transfected, preferably hepatocytic cell lines and HCV-transgenic mouse models have emerged, that enable some conclusions to be drawn as to possible direct profibrogenic and procarcinogenic mechanisms of certain HCV-proteins, irrespective of the host’s immune response. However, only some transgenic mouse strains develop liver damage, pointing to additional genetic predispositions. Furthermore, numerous studies used a highly expressed single gene, or an incomplete set of HCV-genes, a setting that does not mimic human infection, which is usually characterized by moderate viral replication in liver. Finally, expression of a limited set of genes ignores the interactive potential of HCV proteins with each other.

3.21 HCV Core Protein & Non-structural Protein NS5A

HCV core protein represents multifunctional activity. Earlier studies have shown that HCV core protein regulate cellular events and induce hepatic carcinogenesis. It has also been observed that the HCV core protein play a key role in signal transduction of apoptosis, and signal transduction and induction of steatosis and lipid peroxidation.

Shin JY et al. has shown that HCV core protein significantly produced fibrosis related proteins in an in vitro co-culture system. TGF-β1 being recognized as the strongest inducer of fibrogenesis, underwent pronounced expression in media of HSC co-cultured with stable HepG2-core cells compared to that of HSC co-cultured with HepG2 cells. These results suggested that HCV core protein might regulate TGF-β1 expression. In addition, collagen I, which was produced during fibrogenesis, was increased from media of HSC co-cultured with stable HepG2 core cells, again strongly suggesting HCV core protein is closely correlated to fibrogenesis. Taken together it is believed that HCV core protein regulates TGF-β1 and TGFβRII expression to direction of progressive fibrosis.
Connective Tissue Growth Factor (CTGF) is known to be a multifunctional matricellular protein, which has been implicated in wound healing, and several fibrotic diseases including atherosclerosis, pulmonary fibrosis, renal fibrosis, and scleroderma \(^{274,277}\). Although some data showed that CTGF was mainly derived from HSC during liver fibrogenesis \(^{148,278}\), it has been shown that all major cell types in the liver have the potential to produce CTGF according to initial hepatic injury and type of damage \(^{279}\).

Shin JY et al, \(^{274}\) immunocytochemically examined CTGF in HSC co-cultured with either stable HepG2-core cells or with HepG2 cells. In their results, CTGF was predominantly expressed in co-culture of HSC with stable HepG2-core cells than in HSC with HepG2 cells. They noted that CTGF was expressed in both HSC and HepG2-core cells. They also observed that CTGF mRNA and proteins were significantly expressed in the stable HepG2-core cells compared to in the HepG2 cells alone and in normal human liver. All this suggested that HCV core protein up-regulates CTGF in the liver with HCV infection.

So the same authors concluded that: the expressions of α-SMA, TGF-β1, collagen I, TGF-βRII, and MMP-2 were significantly increased in the co-culture of stable HepG2-HCV core with HSC \(^{274}\).

In cell culture the core protein has transforming potential \(^{280}\). It can induce apoptosis. It can interact with the intracytoplasmic TNF-α type 1 and the lymphotoxin β receptors, enhancing their pro-apoptotic signal transduction \(^{281}\), and activate the tumor suppressor p53 \(^{282}\). In addition, HCV core can repress the cell cycle regulator p21 \(^{283}\), and via inhibition of the p38 mitogen activated kinase pathway promote Fas-induced apoptosis \(^{284}\). Taken together, these data rather suggest a pro-apoptotic effect of core protein in hepatocytes, favouring the elimination of infected and potentially transformed cells. On the other hand, anti-apoptotic Bcl-xl can be upregulated \(^{285}\).

While the debate is open as to the predominantly anti- or proapoptotic effect of core protein, the data regarding lipid accumulation in core transfected hepatocytes, one of the second hits that favour hepatic fibrogenesis, are more homogenous. Thus it interacts with apolipoprotein II and reduces microsomal triglyceride transfer protein in vivo, inhibiting assembly and secretion of regular VLDL particles \(^{286}\). As mentioned by Schuppan D \(^{155}\), overexpression of the cytoplasmic located HCV core has been analyzed
in detail. It has been associated with steatosis and hepatocellular carcinoma (HCC). Sensitive C57BL/6 mice with the core transgene driven by the albumin promoter develop hepatic lipid accumulation (steatosis) after 6 months and hepatocellular carcinoma (HCC) after 16 months.287,288

In transfected hepatoma cells core activates the retinoid X receptor alpha which dimerises with the peroxisome proliferator activated receptor alpha to upregulate genes of lipid metabolism like cellular retinol binding protein II and acetyl CoA-reductase.289

Notably, by interaction with mitochondria, core protein induces reactive oxygen intermediates and thus oxidative stress which can induce steatohepatitis.290

The non-structural protein NS5A has also been implicated in HCV pathogenicity. It can compromise the antiviral and hypothetically antifibrotic effect of interferon, obviously, either by repression of protein kinase B or alternate pathways.291 NS5A enhances the acute phase response via activation of NF-kappa B and STAT-3 and as the core, causes oxidative stress.292 In addition NS5A has been implicated in favouring cell cycle progression to the G2/M by binding to the cyclin dependent kinase 1 (cdk1)/cdk2-complex.293

Nunez et al.294 has shown that, intrahepatic COX-2, MMP-2, and MMP-9 over expression is associated with progressive liver disease in chronic HCV infection. They demonstrated that HCV core and NS5A proteins, alone and with the synergistic effect of endotoxin and proinflammatory cytokines (including TNF-α and IL-1β), were able to upregulate COX-2 and MMP-9 gene expression in cultured human hepatic cells. Their study further showed an inducing effect on MMP-9 synthesis in human liver cells is exerted by core protein, but not by NS5A, this induction being partially abrogated by a specific COX-2 inhibitor.

These HCV proteins are known to function as transcriptional transactivators for a wide number of cellular genes.295 It has been reported that core and NS5A proteins are able to activate different transcription factors, such as nuclear factor κB, AP-1, SRE, and STAT-3, suggesting their potential role in upregulation of COX-2 gene expression.292

Alternative candidates transactivated by HCV proteins are PPAR-α ligands, which are able to induce COX-2 expression in hepatocytes.296 Transcriptional regulation by PPAR’s is achieved through PPAR-retinoid X receptor heterodimers and interaction
of core protein with retinoid X receptor α modulates transcriptional activity\textsuperscript{289}. The role of C/EBP-α transcription factor in the regulation of COX-2 expression in hepatocytes has also been described\textsuperscript{297}.

Transgenic mice that express very low levels of the complete reading frame of the HCV polyprotein developed significantly more spontaneous HCC’s after 13 months compared to their nontransgenic controls (5/38 vs 0/16), while transgenic mice expressing significant levels of the HCV structural proteins (Core E1 and E2) only developed HCC in a single case (1/43 vs 0/35)\textsuperscript{298}. While steatosis developed in all animals, inflammation, apoptosis, and histochemical fibrosis were not enhanced in both models, and increased hepatocellular proliferation was only found in the transgenic mice harbouring the complete HCV genome. Matrix gene expression remains to be investigated in these models which should also be challenged, to test how far a second hit can promote both fibrosis and hepatocarcinogenesis\textsuperscript{155}.

3.22 HBV and Liver Injury

The mechanisms of HBV induced liver disease have also been delineated, and some of the representative work is submitted.

Iloeje UH et al,\textsuperscript{299} has shown that progression to cirrhosis in hepatitis B–infected persons is correlated strongly with the level of circulating virus. The risk for cirrhosis increases significantly with increasing HBV-DNA levels and is independent of hepatitis B e-antigen status and serum alanine transaminase level. During a mean follow-up time of 11 years, the 3582 patients contributed 40,038 person-years of follow-up evaluation and 365 patients were newly diagnosed with cirrhosis. The cumulative incidence of cirrhosis increased with the HBV-DNA level and ranged from 4.5% to 36.2% for patients with a hepatitis B viral load of less than 300 copies/mL and 106 copies/mL or more, respectively (\textit{P} < .001). In a Cox proportional hazards model adjusting for hepatitis B e-antigen status and serum alanine transaminase level among other variables, hepatitis B viral load was the strongest predictor of progression to cirrhosis relative risk [95% confidence interval] was 2.5 [1.6–3.8]; 5.6 [3.7–8.5]; and 6.5
for HBV-DNA levels \( \geq 10^4 - <10^5 \); \( \geq 10^5 - <10^6 \); \( \geq 10^6 \) copies/mL, respectively.

i) Apoptosis

As mentioned by Baumert\(^{300}\), the induction of apoptosis is a hallmark of many viruses infecting humans. Although HBV is considered as a non-cytopathic virus\(^{301}\), hepadnavirus induced apoptosis and cytopathic effects have been described in several experimental model systems: 1) a duck hepatitis B variant containing a single amino acid change in the large surface antigen resulting in accumulation of cccDNA resulted in a strong cytopathic effect in hepatocytes in vitro and in vivo\(^{302,303}\).

In this system the level of viral replication and cccDNA formation correlated with cytopathic effects in infected hepatocytes\(^{302}\). 2) Intracellular retention of the HBV large surface protein has been shown to induce apoptosis in cell lines\(^{304}\). In this model overexpression of the large surface antigen resulted in cellular vacuolization and apoptosis of transfected hepatoma cells\(^{304}\). 3) The HBX protein has been suggested to induce apoptosis in both p53-dependent and p53-independent manner\(^{305}\). A viral variant containing two core promoter mutations associate with fulminant hepatitis has been shown to induce apoptosis in primary Tupaia hepatocytes\(^{306}\).

Interestingly in the latter model induction of apoptosis was independent of viral replication, suggesting that viral protein synthesis was sufficient for the virus-induced hepatocyte cell death. Since the two core promoter mutations resulted in two amino acid changes of the HBX protein, the HBX protein may be a potential candidate mediating this effect\(^{306}\).

ii) Other mechanisms of HBV induced cellular injury:

According to experimental models, IFN-gamma counteracts several TGF-beta effects. IFN-gamma displays antifibrotic effects in liver cells via STAT-1 phosphorylation, upregulation of Smad7 expression and impaired TGF-beta signaling. A benefit of 9-month IFN-gamma treatment resulting in decreased fibrosis scores and a reduced number of alpha-smooth muscle actin-positive hepatic stellate cells (HSCs) has
been identified. Approaches opposing profibrogenic activities of TGF-beta may be amenable in chronic liver disease.\textsuperscript{307}

Antiproliferative, pro-apoptotic and immunosuppressive activity effects suggest crucial role of transforming growth factor (TGF)-beta1, metalloproteinase (MMP)-1 and its tissue inhibitor (TIMP)-1 in the pathogenesis of acute liver injury that in some patients precede development of chronic liver diseases and fibrogenesis. Flisiak R et al.\textsuperscript{308} has shown significant correlation between both TGF-beta1 and ALT or AST as well as between TIMP-1 and ALT, AST or bilirubin in acute HBV infection. Elevated baseline levels of both TGF-beta1 and TIMP-1 decreased gradually in consecutive weeks of the disease. TGF-beta1 but not TIMP-1 plasma concentrations were significantly lower in 3rd and 4th week than baseline values. MMP-1 concentration remained on baseline level in the 2nd week of the disease. However in the 3rd week its values increased suddenly but the significant difference in comparison to baseline was observed only in 4th week. Thus there is an important role of TGF-beta1, TIMP-1 and MMP-1 in acute viral hepatitis, that seems to be connected first of all with hepatocytes damage. Their role in extracellular matrix metabolism during acute liver injury needs further evaluation.

Hepatic steatosis occurs frequently in patients with chronic hepatitis B virus (HBV) or chronic hepatitis C virus (HCV) infection. Studies have suggested that steatosis plays an important role as a cofactor in other liver diseases such as hepatic fibrosis, hepatitis, and liver cancer. In contrast to HCV, however, the molecular mechanism by which HBV mediates hepatic steatosis has not been clearly studied. Kim KH et al.\textsuperscript{309} has shown the molecular mechanism by which hepatitis B virus X protein (HBx) induces hepatic steatosis. The increased HBx expression causes lipid accumulation in hepatic cells mediated by sterol regulatory element binding protein 1 and PPARγ, which could be a putative molecular mechanism mediating the pathophysiology of HBV infection. Overexpression of HBx induced hepatic lipid accumulation in HepG2-HBx stable cells and HBx-transgenic mice. It also up-regulated the messenger RNA and protein levels of sterol regulatory element binding protein 1, but not peroxisome proliferator-activated receptor alpha (PPARα). Moreover, the expression of HBx increases PPARγ gene expression as well as its transcriptional activity in hepatic cells, mediated by CCAAT
enhancer binding protein α activation. Finally, HBx expression is able to up-regulate the gene expressions of various lipogenic and adipogenic enzymes in hepatic cells.

What is the role of host genetic factors in chronic HBV infection? Miyazoe S et al. has shown in their study, in HBV carriers, the TNF-α gene promoter polymorphisms were not linked to disease progression. In contrast, allelic frequencies of T and A at positions −819 and −592, respectively, in the IL-10 gene promoter, as well as the frequencies of ATA haplotype at positions −1082/−819/−592 (which is characterized with low capacity for IL-10 production), were significantly higher in asymptomatic carriers than in patients with chronic progressive liver disease. Even after adjusting for individuals positive for anti-HBe, such a relationship could be found between the two groups.

### 3.23 HDV and Liver Injury

Transforming growth factor-β (TGF-β) has been implicated in the pathogenesis of liver disease. TGF-β is involved in liver regeneration and in the fibrotic and cirrhotic transformation with hepatitis viral infection. Hepatitis delta virus (HDV) infection causes fulminant hepatitis and liver cirrhosis. To elucidate the molecular mechanism of HDV pathogenesis, Choi SH et al. examined the effects of HDV-encoded–only protein, the small hepatitis delta antigen (SHDAg), and the large hepatitis delta antigen (LHDAg), on TGF-β– and c-Jun–induced signaling cascades. They found out that: the LHDAg, but not the SHDAg, potentiated TGF-β– and c-Jun–induced signal activation, and the isoprenylation of LHDAg played a major role in signaling cascades. LHDAg synergistically activated hepatitis B virus X protein–mediated TGF-β and AP-1 signaling cascades. In addition, LHDAg enhanced the protein expression level of TGF-β–induced plasminogen activator inhibitor-1. They concluded that: LHDAg may induce liver fibrosis through the regulation of TGF-β–induced signal transductions. This regulation of TGF-β–mediated signaling is accomplished by the isoprenylation of LHDAg, which is a novel mechanism involved in HDV pathogenesis.

Farci P et al. has shown that: high doses of interferon α-2a significantly improved the long-term clinical outcome and survival of patients with chronic hepatitis
D, even though the majority had active cirrhosis before the onset of therapy. Thirty-six patients with chronic hepatitis D who participated in a randomized controlled trial of a 48-week course of high (9 million units) or low (3 million units) doses of interferon α or no treatment were followed for an additional 2 to 14 years.

Long-term survival was significantly longer in the high-dose group than in untreated controls ($P = 0.003$) or in the low-dose group ($P = 0.019$) but did not differ between patients treated with 3 million units and controls. Among surviving patients at 12 years of follow-up, a biochemical response was present in 7 of 12 treated with 9 million units, in 2 of 4 who received 3 million units, and in none of 3 controls. Long-term alanine aminotransferase (ALT) normalization correlated with improved hepatic function and loss of IgM antibody to hepatitis delta antigen (anti-HD). Patients in the high-dose group had a sustained decrease in HDV replication ($P = 0.008$), leading to clearance of HDV RNA and, eventually, hepatitis B virus (HBV) in some patients, as well as a dramatic improvement in liver histology with respect to activity grade ($P = 0.0004$) and fibrosis stage ($P = 0.007$). They documented an absence of fibrosis in the final biopsy of 4 patients with a long-term biochemical response and an initial diagnosis of active cirrhosis $^{312}$. 
Fig. 1 GROSS IMAGE OF A NORMAL AND A CIRRHOTIC LIVER

Gross images of two livers. On the left a normal liver with a smooth surface and homogeneous appearance. On the right a cirrhotic liver with an irregular surface and nodules that give it a heterogeneous appearance.

Fig 2 DIAGNOSIS OF CIRRHOSIS – CT SCAN

Computed tomography findings in compensated cirrhosis. The contour of the liver is irregular, there is obvious splenomegaly and the presence of collaterals indicates portal hypertension and secures the diagnosis of cirrhosis.

Fig. 3 PATHOGENESIS OF LIVER FIBROSIS – STELLATE CELL

The key pathogenic feature underlying liver fibrosis and cirrhosis is hepatic stellate cell activation. Hepatic stellate cells (also known as Ito cells or perisinusoidal cells) are located in the space of Disse between hepatocytes and sinusoidal endothelial cells (that normally are fenestrated). Normally, hepatic stellate cells are quiescent and serve as the main storage site for retinoids (vitamin A).

Fig. 4 Low magnification scanning electron micrograph of the sinusoidal endothelium from rat liver showing the fenestrated wall. Notice the clustering of fenestrae in sieve plates. Scale bar, 1 µm.

Fig. 5 Fibrogenic Cascade

Most forms of liver damage result in hepatocyte injury followed by recruitment of cells and cytokines of inflammation, which, in turn, leads to activation of hepatic stellate cells.

Stellate cell activation is a key pathogenic feature that signifies liver fibrosis and cirrhosis. It includes initiation and perpetuation phases. Key phenotypic features of perpetuation include: proliferation, production of extracellular matrix, contractility, loss of retinoids, and secretions of cytokines and peptides.

ET, endothelin; MCP, monocyte chemotactic protein; MMP, matrix metalloproteinase; PDGF, platelet derived growth factor; TGF, transforming growth factor.


In the normal sinusoid (left), quiescent stellate cells produce little or no endothelin-1 (ET-1), whereas nitric oxide (NO) production by the endothelium is normal. After liver injury (right), stellate cells become activated and produce increased quantities of ET-1; moreover NO production by sinusoidal endothelial cells is reduced. In addition, stellate cell activation, leads to greater expression of smooth muscle proteins. The net effect is enhanced stellate cell contractility and resultant sinusoidal constriction that leads to an increased resistant in sinusoidal blood flow and portal hypertension.

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Chapter 4

LIVER BIOPSY

&

HEPATIC

HISTOPATHOLOGY
Liver Biopsy

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4.1 History

Paul Ehrlich in Germany is credited with the first liver biopsy in 1883\(^1\), and subsequently liver biopsy for diagnostic purposes was reported in 1923\(^2\). In the 1930’s two series were published on the role of liver biopsy in evaluating acute and chronic liver disease\(^3,4\). The procedure became more popular and practical after Menghini reported a quick, “one-second needle biopsy of the liver” in 1958\(^5,6\).

Before Menghini, the previous technique was cumbersome and required on average 6 to 15 minutes of intrahepatic phase making it difficult and probably unsafe\(^5,7\).

With this fundamental modification in technique, the liver biopsy for histologic examination has been central to the investigation of hepatic disease for the last 50 years\(^8-10\). Despite being an invasive procedure, the liver biopsy has become widely used\(^11,12\) in clinical practice, because of the low mortality (0.01-0.17\%), and the relatively low morbidity of the procedure\(^12\).

4.2 Introduction

The liver biopsy is an important diagnostic tool and helps make important therapeutic decisions in acute and chronic liver disease\(^13,14\). It is usually the most specific test to assess the nature and severity of liver diseases\(^7\); and has been considered the “gold standard”\(^13,14\). According to Cholongitas et al,\(^13\) in chronic hepatitis C, histopathologic examination is considered mandatory for grading (necroinflammatory activity) and staging (fibrosis) in most patients\(^16,17\); including patients with persistently normal aminotransferase levels\(^18,19\), and for evaluating steatosis, all histologic features that affect the natural history, prognosis, and therapeutic outcome\(^20-22\). In chronic hepatitis B the same applies\(^13,23\).

In addition to chronic hepatitis B & C, some of the other indications of liver biopsy include diagnosis and evaluation of: nonalcoholic steatohepatitis, alcoholic liver disease, autoimmune hepatitis, hemochromatosis, Wilson’s disease, primary biliary
cirrhosis, cholestatic liver disease, fever of unknown origin, liver mass, status of liver post transplantation and of donor pre-transplantation.

The status of liver biopsy is being challenged by noninvasive tests for the evaluation of fibrosis and its value called into a question owing to variable specimen size and other factors.

The size of the liver biopsy specimen which varies between 1 and 3 cm in length and between 1.2 and 2 mm in diameter, represents 1/50,000 of the total mass of the liver. Usually for the evaluation of diffuse liver disease, a specimen of 1.5 cm in length is adequate for a diagnosis to be made, but some authors claim that a specimen of 2.5 cm represents the best optimal length. The number of portal triads present in the specimen is important; most hepatopathologists are satisfied with a biopsy specimen containing at least six to eight portal triads; but in cases of chronic liver disease in which the extent of injury may vary among portal triads, and due to this reason, one review recommends 11 complete portal tracts. All the needles currently used for liver biopsy usually provide an adequate specimen. Specimens obtained with standard thin-bore or spring-loaded needles measure between 1.4 and 1.8 mm in diameter, and those obtained with Menghini or Trucut needles measure up to 2 mm in diameter.

Advances in medical technology at the molecular level such as markers of liver fibrosis, improvements in imaging modalities, together with advances in drug therapy and refinements in surgical techniques, such as, liver transplantation, have greatly influenced the diagnosis and management of hepatic disease and as a consequence the role of liver biopsy is also evolving.

### 4.3 Indications of liver biopsy

The indications of liver biopsy are outlined in the table. Even for patients in whom serologic tests point to a specific liver disease, a liver biopsy can give valuable information regarding staging, prognosis, and management.

According to Bravo AA, in patients with chronic hepatitis C infection, not only is there a poor correlation between symptoms or levels of serum alanine aminotransferase and histologic features of the liver, but also patients with completely
normal levels of liver enzymes may also be found to have clinically significant fibrosis or cirrhosis on biopsy.\textsuperscript{18,19,28}

If the patient has mild disease and is infected with genotype 1a or 1b of hepatitis C virus, a decision may be made to defer treatment, or the treatment may be stopped if there are significant side effects. Conversely, if the patient has moderate to advanced disease, treatment will most likely be offered. The finding of cirrhosis on liver biopsy will determine the need for further examinations, such as upper GI endoscopy to rule out esophageal varices and screening for hepatocellular carcinoma.\textsuperscript{7}

In alcoholic liver disease, the severity of the clinical symptoms and the degree of liver enzyme elevation correlate poorly\textsuperscript{7,29}.

In patients with alcoholic liver disease, as well as nonalcoholic steatohepatitis, liver biopsy may reveal fatty infiltration of the liver, balloon degeneration, Mallory’s bodies, and hepatocyte necrosis, with or without clinically significant fibrosis or cirrhosis.\textsuperscript{7,29}

In primary biliary cirrhosis, serial liver biopsies help one to study the natural history, monitor the effects of therapy, or identify a recurrence of the disease after liver transplantation.\textsuperscript{7,30,31}

The elucidation of various processes that occur in a transplanted liver— including immune rejection, systemic or infectious complications, drug toxicity, and the recurrence of primary disease—requires a liver biopsy.\textsuperscript{32}

It has been claimed that liver biopsy is able to provide an accurate diagnosis in approximately 90\% of patients with unexplained abnormalities revealed on liver function tests.\textsuperscript{7,33}

### 4.4 Types of liver biopsy needles

There are three general categories of needles used to obtain a percutaneous liver biopsy (the most common of the liver biopsy techniques):\textsuperscript{34}

- Suction needles (Menghini needle, Sure-Cut [modified Menghini needle], Klatskin needle, Jamshidi needle)
- Cutting needles (Trucut needle, Vim-Silverman needle)
- Spring loaded cutting needles that have a triggering mechanisms

Table 6  Indications for liver biopsy

<table>
<thead>
<tr>
<th>Indications for liver biopsy</th>
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<tr>
<td>Grading and staging of chronic hepatitis B and C</td>
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<tr>
<td>Evaluation of abnormal results of biochemical tests of the liver in association with a</td>
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<td>Serologic work up that is negative or inconclusive</td>
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<td>Evaluation of fever of unknown origin, with a culture of tissue</td>
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<tr>
<td>Diagnosis, grading, and staging of nonalcoholic steatohepatitis, alcoholic liver disease, or</td>
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<td>autoimmune hepatitis</td>
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<td>Diagnosis of hemochromatosis in index patient and relatives, with quantitative estimation</td>
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<td>of iron levels</td>
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<td>Diagnosis of Wilson’s disease, with quantitative estimation of copper levels</td>
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<td>Evaluation of cholestatic liver diseases, primary biliary cirrhosis and primary sclerosing</td>
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<td>cholangitis</td>
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<td>Evaluation of the efficacy or the adverse effects of treatment regimens (e.g., methotrexate</td>
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<td>Therapy for psoriasis, effectiveness for therapies for chronic viral hepatitis)</td>
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<tr>
<td>Diagnosis of a liver mass/intrahepatic neoplasms</td>
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<td>Evaluation of the status of the liver post transplantation, or of the donor liver before</td>
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<tr>
<td>transplantation</td>
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<td>Diagnosis and evaluation of systemic disorders, such as, sarcoidosis, lymphoma, acquired</td>
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<td>immunodeficiency syndrome, and amyloidosis</td>
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<tr>
<td>Evaluation of unexplained jaundice, acute hepatitis of uncertain cause, and hepatomegaly</td>
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Adapted from:
The two main types of needle currently being used are the Trucut and the Menghini needles. These two needles use different methods for sampling hepatic tissue. The former, as the name describes, is a cutting needle, whereas the latter uses a suction technique.

These needles come in varying diameters, and the type and gauge of the needle that is optimal for percutaneous liver biopsy has been the subject of several studies. Specimens from the Trucut needles are larger and give more information about liver architecture and may thereby increase the diagnostic yield, however this has undergone re-evaluation. If cirrhosis is clinically suspected, a cutting needle is preferred over a suction-type needle, as fibrotic tissue tends to be fragmented with the latter.

According to Grant A, a large series to look at needle type in relation to complications describes a complication rate of 3.5/1000 for the Trucut needle and 1/1000 for the Menghini needle. Death, serious haemorrhagic complications, pneumothorax, and biliary peritonitis all occurred more frequently with the Trucut needle than with the Menghini needle, whereas puncture of other viscera and sepsis were more frequent with the Menghini needle. Others have reported conflicting results about their relative safety.

Cutting needles, with the exception of the spring-loaded variety, require a relatively longer time in the liver during the biopsy, a factor that may influence the risk of bleeding. Some groups have compared the older Jamshidi suction needle with the Trucut/Vim Silverman cutting needles and found no difference in complication rates.

The theoretical advantages of the Menghini suction technique have been described, the main advantage being that the needle is only in the liver parenchyma for a “second”. This allows less time for the patient to move, thereby minimizing the potential for tearing the capsule.

Although not consistent, a greater risk of bleeding following a biopsy has been observed with larger diameter needles. The needles are considered large when the external diameter is 1.0 mm or more (14-19 gauge), and thin when it is less than 1.0 mm (≥ 20 gauge).
According to Grant A\textsuperscript{12}, one group showed that larger needles produced more bleeding after liver biopsy in anaesthetized pigs. This was statistically significant when comparing 2.1 mm (14 gauge) with 1.6 mm (16 gauge) needles, and also when comparing 1.6 mm with 1.2 mm (18 gauge) and smaller needles\textsuperscript{41}. Human studies on the effect of biopsy needle diameter on complications are rare; however, Forssell and colleagues\textsuperscript{42} could not show any difference in the incidence of intrahepatic hematoma formation when they compared the 1.6 mm modified Menghini needle with a 1.9 mm Jamshidi needle\textsuperscript{12}.

### 4.5 Techniques of liver biopsy

There are currently several techniques available for obtaining the liver tissue\textsuperscript{7,12}:

1. Percutaneous biopsy
2. Transjugular biopsy
3. Laproscopic biopsy
4. Biopsy or fine needle aspiration under ultrasonography or computed tomography
5. Peroperative biopsy

Each of these techniques has its own merits and demerits\textsuperscript{7}. The decision to select a particular technique is based upon available expertise and the particular clinical situation.

### 4.6 Percutaneous liver biopsy

The percutaneous technique for the liver biopsy is the most commonly used procedure and lasts just a few seconds.

Percutaneous liver biopsy may be classified according to the site of entry of the biopsy needle, whether the biopsy is performed in a blind or guided manner, or according to Grant A\textsuperscript{12} whether the biopsy track is plugged after the procedure\textsuperscript{12}.
4.6.1 Transthoracic and subcostal liver biopsy

According to one source Grant A, the patient lies supine for both of the approaches of the procedure. The borders of the liver are usually defined by percussion or visualized by ultrasound. In most instances the intercostal space in the midaxillary line just cephalad to the costal margin is then infiltrated with the local anaesthetic, the biopsy needle is then advanced into the intercostal space. The patient then holds his/her breath in expiration. The subsequent procedure for taking the liver biopsy then varies according to whether the biopsy needle is of aspiration or cutting type.

If the patient has an enlarged liver extending below the costal margin, then the site of entry of the biopsy needle may be subcostal. One view claimed that the complications are slightly more frequent with the transthoracic (4.1%) than the subcostal route (2.7%) but usually this is not the preferred route because of risk of puncturing the gall bladder.

4.6.2 Blind and guided liver biopsy

A blind biopsy is one which is done without imaging of the liver immediately prior to taking the biopsy sample. A guided biopsy is one that is undertaken during imaging of the liver, whether the imaging modality is ultrasound, CT, or MRI scan. Thus guided biopsies should provide bigger specimens, should avoid the puncture of adjacent organs, and should allow the accurate biopsy of focal hepatic lesions, where appropriate. Apart from focal lesions, for diffuse disease, it is the experience of the operator that counts more than the imaging modality, as discussed subsequently.

4.6.3 Ultrasound guided liver biopsy

Ultrasound guided percutaneous liver biopsy is used extensively in the investigation of focal liver lesions, however its use in diffuse liver disease is more controversial.

According to another source, ultrasonography prior to a liver biopsy identifies silent mass lesions (such as haemangioma) and defines the anatomy of the liver and the relative positions of the gallbladder, lung, and kidney. Many practitioners routinely
recommend an ultrasound of the liver prior to performing a percutaneous biopsy. Others perform an ultrasound only in selected patients such as those who have a history of prior upper abdominal surgery, those in whom the point of maximal hepatic dullness cannot be elicited, and those who are suspected of having advanced cirrhosis with possible atrophy of the right lobe of the liver based upon clinical or laboratory grounds.  

In a study that evaluated 222 consecutive liver biopsies by a single operator, ultrasonography was helpful in only 3.6 percent of the patients in whom the biopsy site had to be moved after it had been marked by the percussion technique. A British survey concluded that complications were not avoided by the use of ultrasonography prior to liver biopsy.

Different conclusions were reached in a study of 165 consecutive outpatient liver biopsies, in which ultrasound changed the biopsy site in 21 of 165 patients (13 percent) and led to abortion of the procedure in four patients. Similarly, two large studies demonstrated a lower complication rate and a higher diagnostic yield using ultrasonography guidance.

In one of the reviews, Cholongitas E et al, a total of 5,392 specimens from ultrasound guided procedures and 1,369 specimens from blind biopsy procedures were analyzed. Specimens from ultrasound-guided biopsies were longer than specimens from blind biopsy procedures (20.5 vs 14.4 mm; p=0.021). However ultrasound guided biopsy specimens did not contain significantly more Complete Portal Tracts (CPT’s) than specimens from blind biopsy procedures (8.3 vs 5.3; p=0.13). Specimens obtained using the Menghini needle and ultrasound guidance were significantly longer than specimens obtained with the Trucut and ultrasound guidance (24.4 vs 13.6 mm; p=0.017), and specimens obtained blindly using the Menghini (15.8 mm; p=0.017). There was no significant difference in the length of specimens obtained with the Trucut needle with ultrasound guidance vs blindly.

It has been postulated that ultrasound guided biopsy should reduce complications. As the commonest cause of mortality is bleeding, it follows that the incidence of bleeding should be proportional to the incidence of hematoma formation. The rate of hematoma formation however is unaffected by the use of ultrasound guidance.
One way ultrasound can reduce the incidence of bleeding is potentially it can reduce the number of passes made into the liver. This may be especially important in the context of a shrunken liver where ultrasound may be used to perform the procedure accurately the first time. The increased risk of bleeding associated with multiple biopsy passes has been documented in patients with and without malignancy, and has led to the suggestion that all hepatic tumors should be biopsied by ultrasound or CT guided fine needle aspiration.

Additionally, it is also useful to have a pre-biopsy ultrasound/chest X-ray to rule out any anatomical abnormalities such as Chilaiditi syndrome, where bowel lies between the liver and the abdominal wall, thereby avoiding inadvertent puncture of an adjacent viscus; or a haemangioma in the liver. It is also helpful in patients in whom the liver cannot be easily identified for reasons such as obesity.

### 4.6.4 Plugged liver biopsy

According to the source Grant A, plugged liver biopsy is a modification of the percutaneous approach first described in 1984. It has been advocated as an alternative method for obtaining liver tissue in patients with impaired coagulation where transjugular biopsy is not available.

In this technique a biopsy sample is taken using a Trucut needle in the conventional manner, but only the obturator containing the specimen is removed, leaving the outer cutting sheath within the liver parenchyma. A plastic cannula is then inserted down the sheath and while the breath is still held in expiration, gelatin or gelfoam is injected as the sheath is withdrawn.

### 4.7 Transjugular, transvenous liver biopsy

Disorders of coagulation commonly occur in patients with liver disease and the conventional practice in circumstances where there is significant disturbance of clotting is to avoid percutaneous liver biopsy because of risk of bleeding.
Transvenous liver biopsy was first described in 1964. Then in 1967, the transjugular catheterization of hepatic veins in human subjects was first described, as an access to the biliary tree for cholangiography. In the transjugular liver biopsy the liver tissue is obtained from within the vascular system, it minimizes the risk of bleeding. This is usually done through the transjugular approach; but rarely also done via a femoral approach. It is performed in the vascular catheterisation laboratory with videofluoroscopy equipment and cardiac monitoring because of risk of cardiac arrhythmias as the catheter passes through the right atrium.

According to Bravo A, et al, the internal jugular vein is (usually) cannulated on the right side and a sheath inserted via a Seldinger technique. A 45 cm long catheter is then guided under fluoroscopic control through the right side of heart to the inferior vena cava. The catheter is then loaded with the transvenous biopsy needle and advanced into the hepatic veins and the position checked by injection of contrast medium. The needle is then advanced rapidly 1-2 cm post the tip of the catheter with the patient holding his/her breath and the liver tissue is retained in the needle by aspiration/suction by a syringe attached to the other end of the needle while it is still inside the liver. The duration of the procedure is between 30 to 60 minutes.

According Bravo A, adequate tissue for histologic diagnosis can be obtained in 80-97% of the patients in centers where a large number of transjugular biopsies are performed. The tissue specimen is usually 0.3 to 2 cm long, and the procedure generally requires multiple passes. The samples obtained are small and fragmented, a disadvantage of the technique that may be obviated with newer generation technology. However, in centers where transjugular biopsies are performed regularly, larger samples with fewer passes are obtained. A transjugular liver biopsy may be performed at the same time as the placement of a transjugular intrahepatic portosystemic shunt (TIPS).

The rate of complication associated with transjugular liver biopsy ranges from 1.3% to 20.2%; and the mortality ranges from 0.1% to 0.55%.

Complications of transjugular liver biopsy include: neck haematoma, transient Horner’s syndrome, transient dysphonia, cardiac arrhythmias, pneumothorax, abdominal pain, fistula formation between hepatic artery or portal vein or the biliary tree, and the perforation of the liver capsule (especially in small cirrhotic livers).
Heterogeneity of the liver disease and interobserver or intraobserver variation has not been evaluated in transjugular liver biopsy\textsuperscript{13}.

One review\textsuperscript{13}, analyzed 15 studies of transjugular liver biopsy with 1,389 TJLB specimens; (mean = 2.5 passes per patient), the mean ± SD length was 13.5 ± 4.5 mm (13 of 15 studies), and the mean ± SD number of CPT’s was 6.8 ± 2.3 (6 studies). According to the review\textsuperscript{13}, only the author’s own large series of TJLB (n=326)\textsuperscript{58} detailed the number of passes (n=3), needle size (Trucut 19 gauge), length (mean 22.5 mm), number of CPT’s (mean 8.7), and fragmentation (median=5)\textsuperscript{58}, most studies on the other hand were small series, and were deficient in one or estimates.

One study\textsuperscript{59} compared Trucut (18 gauge) and Menghini type (16 gauge) needles and found that using the Trucut resulted in significantly larger specimens (12 vs 7 mm, p = <0.05)\textsuperscript{58}.

The authors\textsuperscript{13} concluded that the quality of TJLB, as well as interobserver and intraobserver variation, requires further study because this method allows multiple passes (to obtain adequate samples) with far less likelihood of increasing complications\textsuperscript{13,54,60,61}.

### Indications for Transjugular Liver Biopsy

- Severe coagulopathy
- Massive ascites
- Massive obesity
- Suspected vascular tumor or peliosis hepatis
- Need for ancillary vascular procedure (e.g. TIPS or venography)
- Failure of percutaneous liver biopsy

Table 7. **Indications for transjugular liver biopsy**


How does TJLB compare with PLB? In this study\textsuperscript{58}, 326 consecutive TJLB specimens, using three passes (19 gauge Trucut) were compared with 40 consecutive
PLB specimens using (15 gauge Menghini) needle. The study concluded that TJLB with 3 passes is adequate for histologic diagnosis, with 89% of specimens being either ≥ 15 mm or having ≥ 6 portal tracts. Although like PLB, adequate sampling remains a limitation for staging and grading of chronic hepatitis with TJLB, nonetheless, the feasibility of TJLB is comparable to PLB in this respect.  

4.8 Laparoscopic liver biopsy

Laparoscopic liver biopsy is well established, however its use varies between centers. For instance, it is ideal in patients who have a combination of a focal liver lesion and a coagulopathy, where a histologic diagnosis is essential in the management of that patient. Some centers in USA perform laparoscopic liver biopsy on an outpatient basis and in some Japanese centers more than 50% of liver biopsies are performed laparoscopically.

However, generally, the use of laparoscopic liver biopsy by gastroenterologists has declined in favour of less invasive radiologic procedures, and usually the procedure is performed by surgeons. The indications for and contraindications to the laparoscopic liver biopsy are outlined in the table.

According to Bravo A, the complications include perforation of a viscus, bleeding, hemobilia, laceration of the spleen, leakage of ascitic fluid, hematoma in the abdominal wall, vasovagal reaction, prolonged abdominal pain and seizures.

4.9 Fine Needle Aspiration Biopsy

According to one source, Lundquist in 1971 demonstrated that fine needle aspiration compared favourably with the final histologic diagnosis based on surgical specimens. Patients with focal hepatic lesions are good candidates for fine needle aspiration biopsy, especially if they have a history of cancer.

The diagnostic accuracy ranges from 80 to 95 percent, and is substantially affected by the expertise of the cytopathologist. Cytologic findings that are negative for cancer, however, do not rule it out.
Indications for and Contraindications to Laparoscopic Liver Biopsy

**Indications**

Staging of cancer

Ascites of unclear cause

Peritoneal infections

Evaluation of an abdominal mass

Unexplained hepatosplenomegaly

**Contraindications**

**Absolute**

Severe cardiopulmonary failure

Intestinal obstruction

Bacterial peritonitis

**Relative**

Uncooperative patient

Severe coagulopathy

Morbid obesity

Large ventral hernia

Table 8. Indications for and contraindications to laparoscopic liver biopsy


Fine needle aspiration biopsy of the liver is performed under ultrasonographic or CT guidance. Although it is usually reserved for focal hepatic lesions; limited data suggest that diagnostically useful material can be obtained with automatic spring-loaded
biopsy needles guided by ultrasound in over 95% of patients ⁶⁶, including those with diffuse liver disease.

Fine needle aspiration biopsy is associated with a low risk of seeding of the needle track with malignant cells, and is generally a very safe procedure, even in patients with haemangiomas, and echinococcal cysts ⁷,⁶⁷,⁶⁸.

4.10 Complications of percutaneous liver biopsy

The indications for, and methods of liver biopsy have changed over the last few years ⁶⁹, with the advent of new imaging techniques, better facilities at patient monitoring, and new indications for liver biopsy such as liver transplantation ⁷⁰. All invasive procedures have a mortality rate associated with them, and consequently the benefits of obtaining liver sample for histology should always be weighed against the possible morbidity and mortality of the procedure ¹².

4.10.1 Mortality

According to Grant A, the reported mortality from liver biopsy varies considerably. This is partly because most of the larger series reporting liver biopsy complications have been retrospective ³⁶,⁵⁵. The overall mortality rate also varies according to the center in which the liver biopsies were performed—for example, an audit of liver biopsies performed in the UK district general hospitals the death rate was between 0.13% to 0.33% ¹⁰,¹². And in the Mayo Clinic the mortality from fatal haemorrhage after percutaneous biopsy was 0.11% ³⁸.

A generally accepted mortality rate in standard textbooks is between 0.1% and 0.01% ¹¹,¹².

According to Reddy KR, et al, ⁶ although the liver is a rich vascular organ with an intricate meshwork of supply and drainage, the complications associated with percutaneous liver biopsy are fortunately rare. This may be related to the elasticity of the liver parenchyma itself or the elasticity of the biopsy track collapsing down after core has been taken, or to the presence of high concentration of clotting factors within the liver substance ⁷¹. It should, however, be borne in mind that during a blind percutaneous liver
biopsy, the liver is not the only structure to be punctured and the skin and subcutaneous tissues (and occasionally other organs) can bleed. Thus, peripheral indexes of clotting must still be taken into consideration\textsuperscript{12}.

The most dreaded of all the complications is haemorrhage, which can manifest as free intraperitoneal bleed, intrahepatic or subcapsular hematoma, or hemobilia. Sixty percent of complications occur within two hours after the procedure, and 90\% within 24 hours\textsuperscript{36,72}. Fatal complications typically occur within 6 hours after a liver biopsy. A hospitalization rate of 1.4\% to 3.2\% for the management of complications following a liver biopsy has been reported, with pain or hypotension as the predominant cause\textsuperscript{6,27,73}.

\subsection*{4.10.2 Causes of mortality}

According to Grant A, et al.,\textsuperscript{12} the main cause of mortality after percutaneous liver biopsy is intraperitoneal haemorrhage as shown in a retrospective Italian study of 68,000 percutaneous liver biopsies in which all six patients who died did so from intraperitoneal haemorrhage\textsuperscript{36}. Three of these patients had had a laparotomy, and all had either cirrhosis or malignant disease, both of which are risk factors for bleeding\textsuperscript{38,71}. Other serious complications responded to treatment; puncture of viscera was never followed by serious complications. Other series have shown, however, that puncture of the gall bladder followed by biliary peritonitis is a recognized cause of death\textsuperscript{10}.

As the main source of mortality after percutaneous liver biopsy is haemorrhage, it is reasonable to assume that improvements in mortality rates can be made if the clinician understands the risk factors for bleeding, recognizes bleeding promptly and aggressively resuscitates the patient. It has also been suggested that patients who bleed significantly (i.e., patients whose haemoglobin falls to >20g/l or who become haemodynamically unstable) should be considered for either laparotomy or therapeutic angiography if the bleeding does not stop with transfusion alone\textsuperscript{10,12}; as well as those patients who are suspected to have biliary peritonitis should also have an early laparotomy\textsuperscript{12}.

\subsection*{4.10.3 Morbidity}

According to Grant A,\textsuperscript{12} like mortality, the overall morbidity from percutaneous
liver biopsy varies, and is difficult to ascertain as most studies are retrospective and therefore symptoms such as post-biopsy pain requiring simple analgesia, or transient vasovagal drop in blood pressure, are not recorded. Similarly, there is no agreement about the division into major and minor complications, and whether complications such as asymptomatic post-biopsy intrahepatic/subcapsular haematoma should be included in the figures.

A morbidity rate of 5.9% for patients suffering from minor complications after liver biopsy has been reported.

### 4.10.4 Intraperitoneal haemorrhage

According to Reddy KR, et al., significant intraperitoneal haemorrhage is the most serious of all complications and often becomes apparent within the first 2 to 3 hours after the procedure. In a large study of the complications of 68,276 liver biopsies, haemoperitoneum occurred in 0.32% of patients. Significant haemorrhage (indicated by a drop in haemoglobin of >20 g/l) occurred in 0.35 to 0.5% of all procedures. Free intraperitoneal haemorrhage may be related to a laceration sustained during deep inspiration as the biopsy is performed, or to a penetrating injury of a branch of the hepatic artery or portal vein. However, the overall reported incidence ranged from 0.03% to 0.7%.

Delayed free intraperitoneal haemorrhage after a 24 hour period is infrequent and unpredictable. The interval before delayed haemorrhage after liver biopsy has ranged from 36 hours to 18 days, and delayed haemorrhage is associated with a high mortality rate. Haemorrhage after liver biopsy is more common in patients who are elderly, have cirrhosis or a malignant lesion, or undergo multiple passes.

Hypotension or tachycardia following a biopsy, particularly when associated with abdominal pain, is usually the consequence of haemorrhage. Ultrasonography, preferably performed at the bedside, or CT may readily demonstrate a free intraperitoneal collection. Measures to improve haemodynamic status may be sufficient, blood or blood products should be administered. Qualitative platelet abnormalities must be kept in mind. Success with a conservative approach is apparent within a short period of time. If haemodynamic
instability persists despite aggressive resuscitative measures during a couple of hours, alternative measures should be pursued.

According to Reddy KR, angiography is the preferred procedure, and experienced angiographers are able to embolize a peripheral bleeding vessel and any associated arteriovenous fistula. Some of the agents used for embolization include gelatin sponges, autologous blood clot, bucrylate, polyvinyl alcohol sponges, detachable balloons, steel coils, and platinum microcoils. The major complications of transarterial embolization include ischemia or infarction of the liver, gall bladder, or pancreas; rates range from 4% to 15%. Minor complications include fever, bacteremia, pain and elevation of transaminases. Surgical exploration is preferred, if haemodynamic instability persists despite aggressive measures, for a relatively rapid access to the bleeding site/vessel.

4.10.5 Intrahepatic and subcapsular haematomas

According to Reddy KR, intrahepatic and subcapsular haematomas are noted frequently when ultrasonography is performed after a liver biopsy. The incidence of these haematomas following liver biopsy ranges from 17% to 23%. These are often asymptomatic and the frequency is similar in both laparoscopically guided and “blind” liver biopsies. The duration of bed rest after a liver biopsy, ranging 6 to 24 hours, does not appear to influence the incidence of haematoma. A rapid enlargement of the liver associated with tenderness may suggest subcapsular or intrahepatic haematoma. Fever and leukocytosis may be present, and a rare occurrence is biliary obstruction caused by a large haematoma impinging on the biliary tree. Conservative treatment is generally sufficient, and angiography may rarely be required to embolize an arteriovenous fistula.

4.10.6 Pain

Pain is probably the commonest complication of liver biopsy occurring in up to 30%, with moderate and severe pain occurring in 3% and 1.5% respectively. After a liver biopsy, approximately one third of patients experience pain in the right upper quadrant or right shoulder. The pain is usually dull and mild, but occasionally may be
sharp and stabbing, and responds to analgesics. It is also of short duration, occasionally lasting few hours. Generally, the long procedure or multiple passes are associated with increased severity of pain. Ongoing severe abdominal pain should alert the physician to the possibility of bleeding or peritonitis 6.

Transient hypotension and vasovagal episodes are common accompaniments to pain, occurring in about 3% of liver biopsies 39, and vasovagal episodes occasionally require the administration of atropine 12, and fluid boluses.

4.10.7 Haemobilia

Hemobilia is a well recognized but infrequent complication of liver biopsy, presenting with a classic triad of gastrointestinal bleeding, biliary colic, and jaundice 86,87. It has an incidence of 0.05% 12. Piccinino et al, 36 reported 4 cases in 68,276 biopsies. Cholecystitis 88, and pancreatitis 89 are infrequent complications of hemobilia. Hemobilia may resolve with conservative treatment, but ongoing or intermittent bleeding requires intervention. Successful treatment is achieved angiographically in 95% of patients 6,86,87.

4.10.8 Miscellaneous complications

Puncture of other viscera occurs infrequently, with an incidence between 0.01% and 0.1% 36. The puncture of lung, colon, kidney, and gall bladder, together with pneumothorax, pleural effusion, and subcutaneous emphysema are well recognized complications, which rarely require intervention 90.

Other complications include sepsis, reaction to anaesthetic, and breakage of the biopsy needle 12,91.

4.11 Contraindications

Some of the contraindications of liver biopsy have changed overtime due to patient selection, the availability of newer imaging modalities, choice of the technique (transjugular/laparoscopic/ percutaneous), and modern patient care facilities.
Table 9. Complications of percutaneous liver biopsy

<table>
<thead>
<tr>
<th>Complication</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain</td>
<td>(0.056% - 22%)</td>
</tr>
<tr>
<td>Pleuritic</td>
<td></td>
</tr>
<tr>
<td>Peritoneal</td>
<td></td>
</tr>
<tr>
<td>Diaphragmatic</td>
<td></td>
</tr>
<tr>
<td>Haemorrhage</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal (0.03 - 0.7%)</td>
<td></td>
</tr>
<tr>
<td>Intrahepatic and/or subcapsular (0.59% - 23%)</td>
<td></td>
</tr>
<tr>
<td>Haemobilia (0.059% - 0.2%)</td>
<td></td>
</tr>
<tr>
<td>Bile peritonitis (0.03% - 0.2%)</td>
<td></td>
</tr>
<tr>
<td>Bacteremia</td>
<td></td>
</tr>
<tr>
<td>Sepsis (0.088%) and abscess formation</td>
<td></td>
</tr>
<tr>
<td>Pneumothorax and/or pleural effusion (0.08% - 0.28%)</td>
<td></td>
</tr>
<tr>
<td>Haemothorax (0.18% - 0.49%)</td>
<td></td>
</tr>
<tr>
<td>Arteriovenous fistula (5.4%)</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous emphysema (0.014%)</td>
<td></td>
</tr>
<tr>
<td>Reaction to anaesthetic (0.029%)</td>
<td></td>
</tr>
<tr>
<td>Breakage of the needle (0.02% - 0.059%)</td>
<td></td>
</tr>
<tr>
<td>Biopsy of other organs</td>
<td></td>
</tr>
<tr>
<td>Lung (0.001% - 0.014%)</td>
<td></td>
</tr>
<tr>
<td>Gall Bladder (0.034% - 0.117%)</td>
<td></td>
</tr>
<tr>
<td>Kidney (0.029% - 0.096%)</td>
<td></td>
</tr>
<tr>
<td>Colon (0.0038% - 0.044%)</td>
<td></td>
</tr>
<tr>
<td>Mortality (0.0088% - 0.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Sources: [6,36,39,73,74,76,81,82].

For a patient with suspected chronic liver disease/cirrhosis, who has one or more of the features of obesity, coagulopathy, and ascites, a transjugular approach is preferred in most institutions\(^6\). And, laparoscopy is preferred in patients with ascites of unknown cause or in whom a mass lesion is suspected\(^6\). Liver biopsy is a safe procedure when performed by experienced operators. Froelich et al, \(^92\) noted a lower complication rate for physicians who performed more than 50 biopsies a year\(^7\).

### 4.11.1 The uncooperative patient

In percutaneous liver biopsy it is essential that the patient is cooperative as an untoward movement when the biopsy needle is in the hepatic parenchyma can lead to a tear of the parenchyma and/or the capsule and subsequent torrential bleeding. If the patient is anxious, then the use of midazolam as sedation and/or meperidine\(^6\) can be considered with no increased risk\(^93\). If the patient remains uncooperative and the benefit of obtaining liver histology outweighs the risk to the patient, then liver biopsy under general anaesthesia should be considered\(^12\).

### 4.11.2 Abnormal coagulation indices

According to Grant A, \(^12\) there is no consensus about the values at which abnormal coagulation indices become contraindications to percutaneous liver biopsy. A number of investigators have shown that the degree of bleeding from the liver puncture site (observed at laparoscopy) bears no correlation to peripheral blood coagulation parameters, when these parameters are modestly increased\(^94,95\).

It has been postulated that this discrepancy in liver bleeding time may be due to the inherent elasticity of the biopsy track collapsing down after the core has been taken, together with the high local concentrations of clotting factors within the hepatic parenchyma\(^71\). It should, however, be borne in mind that during a blind percutaneous liver biopsy, the liver is not the only structure to be punctured and the skin and subcutaneous tissues (and occasionally other organs) can bleed. Thus, peripheral indexes of clotting must still be taken into consideration\(^12\).
### Table 10. Contraindications to percutaneous liver biopsy

<table>
<thead>
<tr>
<th><strong>Absolute</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncooperative patient</td>
</tr>
<tr>
<td>History of unexplained bleeding</td>
</tr>
<tr>
<td>Bleeding tendency</td>
</tr>
<tr>
<td>Prothrombin time ( \geq 3-5 ) sec more than control</td>
</tr>
<tr>
<td>Platelet count (&lt; 50,000)</td>
</tr>
<tr>
<td>Prolonged bleeding time ( \geq 10 ) min</td>
</tr>
<tr>
<td>Unavailability of blood transfusion support</td>
</tr>
<tr>
<td>Suspected haemangioma or other vascular tumor</td>
</tr>
<tr>
<td>Inability to identify an appropriate biopsy site by percussion or ultrasonography</td>
</tr>
<tr>
<td>Suspected echinococcal cysts in the liver</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Relative</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Morbid obesity</td>
</tr>
<tr>
<td>Ascites</td>
</tr>
<tr>
<td>Infection in the right pleural cavity or below the right hemidiaphragm</td>
</tr>
</tbody>
</table>

Adapted from:


Liver biopsy may be helpful in determining the extent of liver damage in patients with haemophilia and the benefits of treatment in those infected with hepatitis C virus. In the absence of factor concentrate inhibitors, liver biopsy is safe if the clotting abnormalities are corrected before and for 24 hours after biopsy.\(^96\)
4.11.3 Prothrombin time

According to Grant A, several large studies have not shown a greater risk of bleeding associated with increased prothrombin time of four seconds above control values, as well as a large retrospective study of percutaneous liver biopsy to date in which the prothrombin time was prolonged by seven seconds.

By contrast, a number of other studies, however, support the widely held belief that a coagulopathy predisposes the patient to bleeding diathesis after percutaneous liver biopsy. The 1991 BSG audit of the biopsy practice in 189 health districts in the United Kingdom showed that bleeding occurred more commonly if the international normalised ratio (INR) was raised, with 3.3% of the bleeds occurring when the INR was between 1.3 and 1.5, and 7.1% occurring when the INR was >1.5. This suggests that about 90% of the bleeds occurred in patients with an INR<1.3 and reinforces the fact that having a normal INR or prothrombin time is no reassurance that the patient will not bleed after the procedure. However, it must be borne in mind that patients with prolonged INR ≥ 1.5 might not have been subjected to liver biopsy in the first place.

4.11.4 Thrombocytopenia

According to Grant A, there is no general agreement at the level at which thrombocytopenia becomes a contraindication to liver biopsy. In the UK the recognized texts recommend that platelet count should be above 80 000/mm³. The Mayo Clinic regards counts as low as 56 000/mm³ to be safe, whereas a survey of mostly US centers showed a preference for platelet counts above 50 000/mm³. The evidence for a cut off value remains scanty and takes no account of the function of the platelets.

The absolute value of the platelet count may not be crucial in determining the risk of bleeding as it is well recognized that even those patients with normal prothrombin times and platelet counts can have haemorrhage from severely deranged bleeding times. Nevertheless, according to Grant A, for a percutaneous liver biopsy the minimum safe limit for a platelet count is 60 000/mm³.

4.11.5 Platelet function/Bleeding time
Some authorities recommend that a bleeding time should be routinely obtained\textsuperscript{27,98}, and an alternative approach considered if it is prolonged beyond 10 minutes\textsuperscript{27}. One group\textsuperscript{12} suggests that BT is seldom if ever measured in UK centers prior to liver biopsy even though the ingestion of aspirin and other non-steroidal anti-inflammatory drugs in the week prior to invasive intervention is a recognized contraindication by several authorities. And to their knowledge, however, there are no convincing data to support this as a contraindication to percutaneous liver biopsy\textsuperscript{12}.

Patients with renal impairment usually have abnormalities of platelet function. According to one small study, patients with end stage renal failure on haemodialysis are at high risk (up to 50%) of haemorrhagic complications after percutaneous liver biopsy, independent of the BT\textsuperscript{99}. This same study suggested that liver transplant recipients with a BT above 10 minutes (upper limit of normal) had a higher incidence of bleeding complications compared with those with a BT below 10 minutes. The sample size, however, was too small to allow any firm conclusions to be drawn\textsuperscript{12}. Several other factors are likely to affect platelet function with or without affecting the BT. A bleeding time may be elevated in patients with cirrhosis despite what appears to be an adequate platelet count and prothrombin time.

In a study conducted at The Royal Free Hospital, within a group of cirrhotic patients, those with abnormal BTs (42%) were more likely to have significantly lower platelet counts, longer prothrombin times and higher blood urea and serum bilirubin than those with normal BTs (58%). The investigators also demonstrated that the bilirubin concentration and the platelet count were independently associated with the BT (although the correlation for the latter was weak, and the raised serum bilirubin may well be just a surrogate marker for the severity of liver disease)\textsuperscript{12,100}. However, this practice has not been uniformly adopted\textsuperscript{101}.

One source recommends,\textsuperscript{34} to check a bleeding time in patients with renal failure, those who are HIV positive, or patients with a history of excessive bleeding despite a normal prothrombin time, partial thromboplastin time, and platelet count; and to this list patients with advanced cirrhosis may be added.
4.11.6 Ascites

According to Grant A, percutaneous liver biopsy in the presence of significant ascites is considered a contraindication in many texts. The reasons are; from the high likelihood of not obtaining a biopsy specimen because of the distance between the abdominal wall and the liver, to the potential severity of underlying liver disease, and increased risk of complications, such as uncontrollable bleeding into the ascites. Although these reasons seem to be sensible, they are not substantiated in randomized, controlled clinical trials. There is evidence, however, to support the fact that CT or ultrasound guided liver biopsy in the presence of ascites does not affect the complication rate. Notwithstanding these studies, generally, liver biopsy is avoided in the presence of tense ascites. However, if strongly indicated, the options include; total paracentesis prior to liver biopsy, image guided biopsy, transjugular liver biopsy, or laparoscopic biopsy.

4.11.7 Cystic lesions

According to Grant A, modern imaging techniques can identify benign cystic lesions of the liver, thereby eliminate the need for biopsy in many cases. Cystic lesions within the liver may communicate with several structures including the biliary tree and therefore pose a risk of biliary peritonitis after biopsy.

Echinococcal cysts have been considered a contraindication to percutaneous liver biopsy because of the risk of anaphylaxis from the dissemination of the hydatid cysts throughout the abdomen. New developments, such as aspiration of hydatid cysts with 19-22 gauge needles under ultrasound guidance has been shown to be safe for both diagnostic and therapeutic purposes.

Quality of Liver Biopsy Specimens
The liver biopsy has an important diagnostic role that helps make important therapeutic and prognostic decisions in acute and chronic liver disease\textsuperscript{13,14}. The histopathological examination has been considered the “gold standard”\textsuperscript{13}. Though the biopsy specimen samples approximately 1/50,000 of the mass of the liver, but is considered reasonably representative of the whole liver pathology\textsuperscript{7}. There have been questions regarding the quality of liver biopsy specimens, and according to Cholongitas et al\textsuperscript{13}, studies have evaluated the following\textsuperscript{13}:

1. What is the optimal size of the liver biopsy specimen required for the accurate description of liver disease?
2. When there is heterogeneity of diffuse liver disease, does the small size of constitute a problem in diagnosis?
3. Does interobserver and intraobserver variation significantly affect the liver biopsy interpretation?
4. Which histopathological scoring system is the most reliable and reproducible?
5. What is an adequate liver biopsy sample?

With the development of noninvasive tests of liver fibrosis, the quality of liver biopsy specimens is further under scrutiny due to the above mentioned factors. Studies have evaluated optimal length\textsuperscript{25,107} and number of portal tracts\textsuperscript{107} for accurate grading and staging in chronic viral hepatitis. Thus, there has been further emphasis on the quality of liver biopsy leading to an accurate interpretation\textsuperscript{13}.

\section*{4.12 Sampling Variability}

Even before the advent of the semiquantitative scoring systems for chronic hepatitis, several studies already have pointed out sampling variability as a major potential limitation of liver biopsy\textsuperscript{108-110}. These studies were published before the development of semiquantitative scoring systems for chronic hepatitis, and underlined the difficulty caused by sampling errors, especially when using biopsy specimens to diagnose cirrhosis\textsuperscript{25}.
Maharaj et al,\textsuperscript{111} by performing 3 transcutaneous biopsies in the same patients using different entry points, reported that in proven cirrhotic patients, a histologic feature of cirrhosis was present in all 3 biopsy specimens of only 50% of the patients. Similarly, Abdi et al,\textsuperscript{110} performed several postmortem biopsies and showed that the diagnosis of cirrhosis could be obtained from one biopsy specimen in only 16 out of 20 cases, but that the performance increased to 100% with 3 biopsy specimens\textsuperscript{25}.

For the diagnosis of chronic active hepatitis, several investigators raised the same problems, although to a lesser extent. Soloway et al,\textsuperscript{108} found major discrepancies in diagnosis in cirrhotic patients with sequential biopsies, they found a 90% degree of consistency in grading individual histologic features of chronic hepatitis including fibrosis. Less favourable results were published by Baunsgaard et al,\textsuperscript{112} who showed a concordance in only 36 of 50 patients when evaluating the fibrosis amount with 2 biopsies in same patients\textsuperscript{25}.

According to Regev et al,\textsuperscript{113} therefore sampling error has been shown to exist in needle liver biopsy, in respect to severity of inflammation,\textsuperscript{110,114,115} degree of fibrosis,\textsuperscript{110,115} and the presence of cirrhosis\textsuperscript{108,110,115-119}.

Lately, Regev et al,\textsuperscript{113} studied 124 patients with chronic hepatitis C who underwent simultaneous laparoscopy-guided biopsies of the right and left hepatic lobes. The slides were randomly divided among two hepatopathologists after coding. Inflammation and fibrosis were scored according to the modified Scheuer system (Batts and Ludwig).

Thirty of the 124 patients (24.2\%) had a difference of at least one grade, and 41 of 124 patients (33.1\%) had a difference of at least one stage between the right and left lobes. In 18 (14.5\%) patients, interpretation of cirrhosis was given in one lobe, whereas stage 3 fibrosis was given in the other. A difference of two stages or two grades was found in only three (2.4\%) and two (1.6\%) patients, respectively. Of the 50 samples that were examined twice, the grading by each pathologist on the second examination differed from the first examination in 0\% and 4\% and the staging differed in 6\% and 10\%, respectively. All observed variations were of one grade or one stage.

The authors concluded that: liver biopsy samples taken from the right and left hepatic lobes differed in histological grading and staging in a large proportion of chronic
hepatitis C virus patients; however, differences of more than one grade or stage were uncommon. A sampling error may have led to underdiagnosis of cirrhosis in 14.5% of the patients.  

One review, compared 5 studies regarding potential heterogeneity of liver disease. The scoring systems used were Knodell, Ishak, and Scheuer. Only 1 study had biopsy specimens of adequate length. In 50 patients with chronic HCV ultrasound-guided PLB of the right lobe (28 ± 11 mm) and left lobe (25 ± 9 mm) showed no difference between grading and staging in the paired biopsy specimens. According to the review, the studies had variations in length and designs. In the other studies, 1 did not document length, 1 had a mean length of only 12.3 mm and 2 evaluated biopsy specimens selected as 15 mm or longer. These 4 studies, however, showed difference in agreement between grading and staging. But in the overall context, the variations of studies documented by the review, appear smaller than the variation in grading and staging of liver disease.

These studies have pointed out one of the major limitations of fibrosis assessment: sampling variation. As shown by these studies, the problem of heterogeneity could be resolved partially by taking several biopsy specimens from the same patient; a procedure that raises major ethical concerns due to an increase in the risk for morbidity and mortality.

In the context of sampling variability, the size of the biopsy specimen must be considered because it is evident that the longer the biopsy specimen, the lower the risk for an erroneous evaluation due to sampling error.

4.13 Specimen size and histological evaluation of grading and staging

According to the review, Holund et al. first studied diagnostic reproducibility relative to specimen size in 100 selected liver biopsy specimens that were
25 mm or longer, and 1 mm or wider in patients with acute or chronic hepatitis or cirrhosis. They concluded that an LB specimen 5 mm long or longer was adequate for diagnosing acute hepatitis but inadequate for chronic hepatitis or cirrhosis. Subsequently, the same group 114 focused on chronic hepatitis, using the same selection criteria (16-gauge Menghini needles with an external diameter of 1.65 mm). Specimens of 15 mm or more were necessary for an accurate diagnosis of chronic aggressive (active) hepatitis.

According to Cholongitas et al13, another study, in patients with chronic HCV, using same methods evaluated 100 liver biopsy specimens. The specimens were 20 mm or longer. The Metavir scoring system was assessed in different lengths: 5, 10, 15, and 20 mm or longer, with the latter as the reference standard for concordance 126. A 10 mm length was adequate for reliable assessment of necroinflammatory activity and fibrosis (weighted k, 0.81 and 0.85, respectively) 13.

Colloredo et al107, studied 161 liver biopsy specimens in patients with chronic hepatitis B and C, using the Ishak scoring system. The biopsy length was ≥ 3 cm and width 1.4 mm. Subsequently the scoring was blindly repeated by reducing the length to 1.5 cm and 1 cm, and width to 1 mm.

Reducing the length resulted in increased diagnosis of mild grade and small stage of fibrosis. Mild grade was diagnosed; 49.7% in > 3 cm, 60.2% in 1.5 cm, and 86.6% in 1 cm long specimens (p<0.001). Similarly, cases staged as having mild fibrosis significantly increased in the shorter specimens: 59% in > 3 cm, 68.3% in 1.5 cm, and 80.1% in 1 cm long specimens (p<0.001)107.

According to the review13, severe grade was diagnosed in 11.8% of liver biopsy specimens that were 3 cm or longer but only 0.6% of liver biopsy specimens that were 1.5 cm long (both were 1.4 mm wide), and severe stage was diagnosed in 11.2% of liver biopsy specimens 3 cm or longer but in only 3.1% of liver biopsy specimens 3 cm or longer but 1 mm wide. A specimen 20 mm or longer and/or containing 11 or more complete portal tracts was necessary for reliable assessment of grading and staging in chronic viral hepatitis. These criteria have been adopted rapidly as optimal standards 13.

The authors concluded that: liver biopsy size strongly influences the Grading and Staging of chronic viral hepatitis. The use of fine needles should be discouraged in this setting107.
However, according to Cholongitas E\textsuperscript{13}, whether a 16-gauge needle (external diameter, 1.65 mm) results in a constant width of 1.4 mm needs to be questioned because other studies using larger needles (14 gauge; external diameter, 2.1 mm) describe a mean ± SD width of 0.9 ± 0.3 mm\textsuperscript{127}. In practice, biopsy width is not uniform because of variable tissue shrinkage and because the plane of section cannot always be through the maximum diameter of the biopsy cylinder\textsuperscript{13}.

In an important study, Bedossa et al\textsuperscript{25}, evaluated the adequacy of liver biopsy samples obtained at least 3 cm from the tumor by using image analysis of 17 surgical specimens following resection, in patients with chronic hepatitis C. They obtained 10,659 virtual liver samples varying from 2.5 to 200 mm in length and a constant 1.22 mm width.

The image analysis of fibrosis was converted to the METAVIR scoring system (the reference METAVIR stage was based on the whole sample, at least 2 x 3 cm), and compared with histopathological system. The coefficient of variation of fibrosis with 15 mm biopsy was 55\%, and for 25 mm was 45\%. Accurate evaluation of fibrosis was achieved in only 65\% of 15-mm-long and 75\% of 25-mm virtual samples, with no significant improvement with longer samples. The conclusion was that a specimen 25 mm was the minimum length for reliable staging\textsuperscript{25}.

4.14 Intra-observer and Inter-observer variation in the histopathological assessment of chronic viral hepatitis

The way in which liver biopsies showing chronic hepatitis are reported is undergoing re-evaluation. A related question is: how the inflammatory and fibrotic changes in these liver biopsies can be semi-quantitatively assessed so that comparisons can be made between groups of patients and the effect of treatment on disease progression studied\textsuperscript{128}. In an attempt to standardize the histological assessment several semi-quantitative scoring systems have been proposed and evaluated.
In one of the studies by Goldin et al\textsuperscript{128}, a blinded trial was done in which 20 cases of chronic viral hepatitis were assessed by five histopathologists, using the Knodell and Scheuer scoring systems (two of the commonly used systems) on two separate occasions. While both systems produced good inter- and intra-observer variation when fibrosis was assessed; the Scheuer system produced slightly higher kappa values.

According to the authors, the Scheuer system also produced considerably better agreement when the severity of inflammation was examined. A multirater kappa analysis confirmed that both systems showed better agreement for fibrosis scores than inflammatory scores. The authors concluded that, while both systems produced reasonable agreement, this was greater using the Scheuer system\textsuperscript{128}.

Westin J et al\textsuperscript{129}, evaluated the interobserver reliability of Ishak scoring system in 95 liver biopsies from patients with chronic hepatitis C virus infection. For three independent observers, the agreement for periportal hepatitis, focal necrosis, and portal inflammation was found in 95\%-96\%. Kappa scores ranged from 0.11 to 0.41 and weighted kappa scores from 0.18 to 0.53.

For staging the authors noted 84\% agreement, kappa scores of 0.26-0.47, and weighted kappa scores of 0.57-0.69.

The authors concluded that: the Ishak system is associated with good interobserver reliability if a deviance of one categorical level in each variable of the system is accepted as agreement. Compared to the Knodell system it provides more detailed information but is less reliable regarding fibrosis\textsuperscript{129}.

In the study by Grønbæk et al\textsuperscript{130}, five specialist histopathologists evaluated 46 liver biopsies from 20 patients treated with interferon-\alpha. Knodell’s and Ishak’s scoring systems, De Groote’s classification and a four level general necro-inflammatory activity score (GNAS) were applied. In addition to kappa statistics, slide by slide analysis was performed. An acceptable slide agreement was defined as eight of ten observer pairs agreed on 80\% of the slides.

The best agreement was seen for Knodell’s and Ishak’s fibrosis scores, De Groote’s classification and GNAS (mean weighted kappa ($\kappa_w$) = 0.49, 0.51, 0.50, and 0.44, respectively). The concordance was substantial regarding cirrhosis (mean $\kappa =0.69,$
and 0.72, respectively), but only moderate in piecemeal necrosis (mean $\kappa$ =0.40, and 0.39, respectively).

Slide by slide analysis showed the highest agreement on Knodell’s fibrosis score and GNAS; only one point of difference in score was to be accepted to obtain ‘eight of ten’ agreement. In contrast, five points of difference were necessary to accept in order to reach the same agreement for Knodell’s total activity score\(^{130}\).

Regev et al\(^{113}\), studied 124 patients with chronic hepatitis C for sampling error and intraobserver variation in liver biopsy. The patients underwent simultaneous laparoscopic – guided biopsies of the right and left hepatic lobes. The sampling variation part has been described earlier.

In the study, formalin fixed paraffin embedded sections of liver biopsies were stained with hematoxylin and eosin and with trichrome. The slides were blindly coded and randomly divided among two hepatopathologists. Inflammation and fibrosis were scored according to the grading (inflammation) and staging (fibrosis) method based on modified Scheuer system. Fifty of the samples were blindly resubmitted to each of the pathologists to determine the intraobserver variation.

Of the 50 samples that were examined twice, the grading by each pathologist on the second examination differed from the first examination in 0% and 4%, and the staging differed in 6% and 10%, respectively. All observed variations were of one grade or stage.

The authors concluded that the intraobserver variation appeared to be low\(^ {113}\).

One of the reviews by Cholongitas et al\(^ {13}\), already cited to earlier, commented on these and other studies\(^ {128-133}\). According to them the studies had variations in design, and were not uniform. One study\(^ {128}\), used samples of longer length (>40 mm). The Scheuer system had excellent results for intraobserver and interobserver agreement, as did the Knodell system for fibrosis but not for inflammatory score. In the other studies, 1 did not document length\(^ {129}\), 3 used samples 10 mm or longer\(^ {130,131,133}\), and 1 used samples 15 mm or longer\(^ {132}\). According to the review\(^ {13}\), the study that included histopathologists with different levels of expertise, duration, and location of practice\(^ {133}\) and had an excellent design only used specimens 10 mm or longer, and, thus, its results may not be applicable to optimal liver biopsy specimens. But the question remained that how many
of the liver biopsy samples obtained in routine practice are ≥ 40 mm. In clinical practice the majority of the samples are between 10-20 mm, and one has to realistically assess the interobserver variation and intraobserver variation in these biopsy samples with the different histopathological scoring systems. Thus, it is ideal to have a longer liver biopsy sample, but in how many patients this ideal is achieved. According to the review\textsuperscript{13}, in fact, agreement increased in relation to length and number of portal tracts.

In one of the excellent studies by Bedossa et al\textsuperscript{25}, the authors investigated the increase in the METAVIR score and the increase in the amount of fibrosis via image analysis. They reported that the increase in the METAVIR stage is associated with a progressive increase in the fibrosis area. Interestingly, this increase of fibrous tissue accumulation is not linear; because for example, F2 has 3-fold more fibrosis than an F0 stage (normal liver), whereas F3 and F4 are respectively, 7- and 12-fold F0. They concluded that if the METAVIR score of fibrosis appears to increase linearly with time, this is in fact corresponds to an exponential accumulation of fibrous tissue within liver \textsuperscript{25}.

Therefore a difference of just one stage variation in liver biopsies may in fact translate into a much more difference in the actual amount of fibrosis within the liver parenchyma, and results in liver biopsy shown being less than accurate interpretation of liver fibrosis.

In the studies described above, “agreement” was defined as variation in one grade or stage. Due to the facts illustrated above, an agreement of one stage may be misleading, and may amount to speculation, as potentially there could be significant difference in the amount of fibrosis between different stages. The semi-quantitative grading systems should also in some way incorporate this difference to over come this fallacy in reporting different stages, as the total accumulation of fibrous tissue within the liver with subsequent stages increases to a great extent, which is not reflected by the 4-5/6 stages of fibrosis.

4.15 Length and the number of complete portal tracts
How the length of the biopsy specimen and the no. of CPT does correlate? Do we always or most of the times get the required no. of CPT’s with adequate liver biopsy, or is there any optimal length in this regard. Thus according to Cholongitas et al\textsuperscript{13}, and based on preceding discussion, the minimum standards for an optimal percutaneous liver biopsy (PLB)\textsuperscript{25,107} for assessing chronic viral hepatitis require longer specimens than before (≥ 20-25 mm vs ≥ 15 mm)\textsuperscript{7}, and more CPT’s than before (≥11 vs ≥ 6-8)\textsuperscript{7}.

Additionally, in a study\textsuperscript{107}, 11 CPT’s were not obtained while evaluating biopsy specimens 1 mm wide, suggesting that 1 mm is an insufficient width\textsuperscript{13}.

A systematic review by Cholongitas et al\textsuperscript{13}, evaluated these characteristics in 32 studies with a collective pool of 10,027 PLB specimens from 8,746 patients. All 32 studies reported length, but only 12 reported the number of CPT’s. Fragmentation was described in 8 studies, but in only 4 the mean no. of fragments given. Five studies did not report the mean length of the biopsy and the range, but just divided them into categories, i.e., longer or longer than a certain length. The mean ± SD length and the number of CPT’s were 17.7 ± 5.8 mm and 7.5 ± 3.4 mm, respectively. According to the review\textsuperscript{13}, importantly the correlation between length and CPT’s was poor (Spearman r = 0.45, p = 0.04).

The review\textsuperscript{13} further goes on to analyze the length of the biopsy sample and the period in which these were obtained. The percutaneous liver biopsy specimens obtained during the 1996-2005 period compared with those obtained before 1996 were significantly longer (19.8 vs 15.7 mm, p = 0.033); and were more frequently obtained with ultrasound guidance (9 vs 2 studies, p = 0.001); and using smaller needles (18 or 19 gauge, 6 vs 2 studies, p = 0.023)\textsuperscript{13}.

The Menghini needle yielded significantly longer samples (19.9 ± 6.6 mm) compared with the Trucut needle (14.3 ± 3.2 mm, p = 0.016), but without a significant difference in the number of CPT’s (7.3 vs 6.9, p = 0.8)\textsuperscript{13}.

Few studies reported the number of complete portal tracts, and length. Even in those studies, which reported both characteristics, the correlation between length and number of complete portal tracts was poor. In addition, comparing the type of needle and
complete portal tracts; although, longer samples were obtained with the Menghini needle as compared to the Trucut needle, again there was no significant difference in the number of complete portal tracts.

4.16 Type of needle

According to the same review \textsuperscript{13}, in their analysis, there were a total of 4,481 Menghini needle biopsies, 4,134 Trucut needle biopsies, and 1,412 of unknown type. The needle size varied from 14 to 19 gauge (median, 16 gauge).

As discussed in the previous section, the Menghini needle yielded significantly longer samples (19.9 ± 6.6 mm) compared with the Trucut needle (14.3 ± 3.2 mm; P = .016), but without a significant difference in the number of CPTs (7.3 vs 6.9; p = 0.8).

What are the elements behind a longer sample obtained by the Menghini needle. The investigators\textsuperscript{13} commented that; the Trucut needle provides a maximum length of sample determined by the notch in the needle shaft (usually 20-25 mm; compared with the Menghini needle in which the length depends on the force of aspiration and operator experience), and also that the Menghini needle is less traumatic as compared to the Trucut needle, this could explain the longer samples obtained with the Menghini needle. Another potential reason could be that more passes were performed with the Menghini needle. However, the data does not support this notion. In 16 of 32 studies that gave such information showed that more than 1 pass was performed in 108 (3.1%) of 3,535 biopsies using the Menghini compared with 199 (12.1%) of 1,646 biopsies using the Trucut\textsuperscript{13}. Thus more passes were performed with the Trucut needle.

4.17 Needle size

Cholngitas et al\textsuperscript{13}, reviewed the impact of needle diameter and gauge on the length and no. of CPT’s. There was no significant difference in length (range,16.3- 20.7 mm) or number of complete portal tracts (range, 4.6-9.7) according to the needle diameter.
Longer biopsy specimens (mean, 20.7 mm) containing a larger number of complete portal tracts (mean, 9.7) were obtained by using 17-gauge needles, but these results were derived from only 3 studies.\textsuperscript{134,135}

PLB specimens obtained by 18- or 19-gauge needles compared with smaller ones had similar mean length (18.4 vs 18.6 mm) but contained more CPTs (8.0 vs 6.0); however, this difference was not significant.

The Menghini and Trucut needles were compared only in studies with 14-, 15-, and 18-gauge needles. Liver biopsy specimens were longer with the Menghini needle than the Trucut needle across all 3 gauges mentioned, the 14-gauge Menghini (23 vs 15.5 mm; \( P = .18 \)), and 15-gauge Menghini (21 vs 14 mm; \( P = .47 \)), and significantly longer by using 18-gauge Menghini needle as compared to 18-gauge Trucut needle (26 vs 12.8 mm; \( P = .012 \)).\textsuperscript{13}

### 4.18 Thin needle vs large needle for assessment of diffuse liver disease

According to the review,\textsuperscript{13} one study\textsuperscript{136} compared the Menghini thin needle, 20 and 21 gauge, with the conventional Menghini large needle, 17 gauge, in patients with similar indications for biopsy for histological diagnosis. From 258 patients, 343 biopsy samples were obtained, excluding the Ishak stages 5 and 6. A 17-gauge needle used by surgeons using several passes for 28 biopsies (17Gs); single-pass percutaneous for 79 biopsies using a 17-gauge needle (17Gp); and ultrasound guidance with a 20-gauge needle in 88 biopsies (20Gp) and a 21-gauge needle in 80 biopsies (21Gp). Astonishingly, the authors found that specimens in the 20Gp group, compared with specimens in the 17Gp group, were longer (29.8 vs 25.3; \( P < .05 \)) but contained fewer portal tracts (6.7 vs 9.7). An insufficient sample was obtained in 4 cases in the 20Gp group, and in only 1 in the 17Gp group. The authors concluded that 20-gauge needle (20Gp) could be a reliable alternative for patients with diffuse liver disease and contraindications for large-needle, 17 gauge needle (e.g., 17Gp) percutaneous biopsy.
Another study\textsuperscript{137}, examined the reliability of thin-needle biopsy for grading and staging in chronic viral hepatitis: 59 patients underwent thin-needle biopsy (20-gauge, 0.9-mm needle) and 41 underwent large-needle biopsy (17-gauge, 1.4-mm needle). Two independent pathologists using the Ishak scoring system read all samples first separately and then together.

According to the review\textsuperscript{13}, in thin-needle specimens, severe fibrosis (stage 5) and cirrhosis (stage 6) tended to be underestimated, otherwise no significant difference was found for grading and staging between thin-needle and large-needle specimens. However, the limitations of the study were that the thin-needle and large-needle samples were not paired, there was no randomization, and there may have been a bias because there was a significantly lower platelet count in patients who had undergone thin-needle biopsy that likely represented more advanced liver disease and/or cirrhosis, with a greater prevalence of higher fibrosis stages in patients, but still in thin needle biopsy specimens severe fibrosis and cirrhosis were underestimated\textsuperscript{13}.

According to Cholongitas et al\textsuperscript{13}, these limitations were overcome in a study in which biopsy specimens were obtained through the same puncture site from 149 consecutive patients with chronic HCV\textsuperscript{134}. Two histopathologists evaluated the paired thin-needle (0.8 mm) and large-needle (1.2 mm) samples using the Ishak scoring system. Liver biopsy samples were considered adequate if they were 10 mm or longer, contained 4 or more portal tracts, and were not too fragmented.

Large-needle specimens were significantly longer than thin-needle specimens (21.2 vs 12.2 mm; $P < .001$) and less fragmented (11% vs 42%; $P < .001$) and considered adequate more frequently (94% vs 55.7%; $P < .001$). Comparison of the 83 paired and adequate specimens showed that in thin-needle specimens, fibrosis and all 4 categories of necroinflammatory activity were underscored. Finally, thin-needle biopsy resulted in under-staging of cirrhosis (2 of 3 biopsy specimens with stage 5/6). Similar results were obtained with the METAVIR and Scheuer scoring systems. The authors concluded that thin-needle biopsy should be avoided for grading and staging in patients with chronic HCV\textsuperscript{13}. 
4.19 Center experience

According to the review, liver biopsy samples were longer in studies with 100 or more percutaneous liver biopsies (PLB’s) than those with fewer than 100 PLBs (20.4 mm vs 16 mm; \( r = .026 \)). However, this difference was not significant for the number of CPTs (8 vs 7.3; \( p = .7 \)). In these circumstances, even ultrasound guidance did not help to obtain longer specimens (ultrasound-guided vs non-ultrasound guided, 17.9 vs 13.6 mm; \( p = .19 \)).

Specimens obtained with Menghini needles were significantly longer in studies with 100 or more PLBs than those with fewer than 100 PLBs (24 vs 16.1 mm; \( p = .005 \)), in contrast with studies in which Trucut needles were used.

4.20 Transjugular liver biopsy

TJLB has been considered a second grade biopsy owing to the small specimen size and increased fragmentation compared with PLB. TJLB, nevertheless, offers certain advantages; it is being used in circumstances where PLB is avoided or contraindicated, and offers the possibility of using multiple passes without increasing complications.

In the author’s review, overall, 1,389 TJLB specimens (15 studies) were evaluated (mean, 2.5 passes per patient); the mean ± SD length was 13.5 ± 4.5 mm (13 of 15 studies), and the mean ± SD number of CPTs was 6.8 ± 2.3 (6 studies). Most studies described small series.

According to the authors, only their series of TJLB (n = 326) detailed the number of passes (n = 3), needle size (Trucut 19 gauge), length (mean, 22.5 mm), number of CPTs (mean, 8.7) and fragmentation (median, 5). Only 1 study compared Trucut (18 gauge) and Menghini-type (16 gauge) needles and found that using the Trucut resulted in significantly longer specimens (12 vs 7 mm; \( p < .05 \)).

The authors were of the view that with a mean of 2.5 passes, the biopsy specimens were on average only 4.2 mm shorter compared with PLB (13.5 mm vs 17.7 mm, respectively), and, it is important to note, contain almost the same number of CPTs.
(6.8 vs 7.5, respectively), which is similar to the difference between Trucut and Menghini needles. In their study of TJLB58, 89% of specimens were either ≥ 15 mm long or had ≥ 6 CPT’s. The authors58, concluding that although adequate sampling remains a limitation with TJLB for staging and grading of chronic hepatitis, TJLB is comparable to PLB in this respect58.

According to the review13, heterogeneity of liver disease and interobserver or intraobserver variation have not been evaluated in TJLB.

Quality of TJLB requires study, for all the estimates of an adequate liver biopsy sample, because this method allows multiple passes (to obtain adequate samples) with far less likelihood of increasing complication rates60,61.

The authors commented that, TJLB could be an alternative and safe approach to obtain samples of adequate size and a reliable assessment of liver histologic features13.

### 4.21 The adequate liver biopsy sample

So what is the ideal liver biopsy sample in texts and in practice, and how many times one gets the standard PLB sample, and if not always, then what is the trade off. According to the review cited extensively13, the minimum standards for an optimal percutaneous liver biopsy25,107 for assessing chronic viral hepatitis require longer specimens than before (≥20-25 vs ≥15 mm)7 and more CPTs than before (>11 vs >6-8)7. Additionally, in 1 study107, 11 CPTs were not obtained when evaluating masked biopsy specimens 1 mm wide, suggesting that 1 mm is an insufficient width.

These new high standards would result in liver biopsy being less delusive. Thus as evidenced in a study by Goldin et al128, there was less interobserver and intraobserver variation with biopsy specimens that were 40 mm or longer, i.e., a large specimen. In addition, if the new standards are met, potential heterogeneity of liver disease is not significant138.

However, according to the review13, a study136, showed that all methods of LB resulted in an insufficient sample size in a significant proportion of patients: 42% of PLBs with a large 17-gauge needle contained 10 or more portal tracts. Only the surgically obtained LB specimens with multiple passes provided adequate liver samples in a very
high proportion of cases. Using a thin needle allows multiple passes without increasing complications, this advantage is overcome by its low diagnostic performance 134.

Although specimens obtained with Menghini needles are significantly longer than those obtained with Trucut needles (19.5 vs 14.3 mm; \( P = .01 \)) the number of CPTs was no different. A new Trucut needle with a larger notch (at least 30 mm) may overcome this restraint but could result in more complications 13.

In addition to length, for a fully representative liver biopsy sample, the number of complete portal tracts are also important. There is no uniform consensus on the definition of a complete portal tract. The study by Rocken et al 136, for PLBs, similar to the cited author’s study for TJLBs 58, used the definition of Crawford et al 127, for CPTs: complete circumference with at least 2 portal structures within them. Colloredo et al 107, on the other hand, considered CPTs as only the portal triads with complete circumference, and partial portal tracts were those incompletely represented (usually at the margin of the specimens). Moreover, it is recognized that biopsy specimens obtained at the periphery of the liver more frequently contain only a hepatic arterial branch and bile duct, missing the portal vein 127. These portal dyads with a complete circumference are CPTs in the normal liver. In addition, fragmentation will reduce number of CPTs if the break occurs through them 13. This may explain in part why poor correlation was documented between length and CPTs (although still statistically significant, \( r = 0.45; P = .04 \)) 13.

However, more than 1 pass is likely to be needed to obtain a PLB specimen of adequate (ideal) size, which has the potential to increase the complication rate, which increases with needle size and number of passes 12,38,139. For this reason, the clinical applicability of the histopathologic requirement for larger liver biopsy specimens has to be explored critically.

In this systematic review 13, comprising all documented series of PLB in the literature, the liver biopsy specimens had an average length and number of portal tracts well below the published minimum sample size requirements 25,107 in more than half the cases. Thus according to the review 13, it is surprising that only 2 of 147 studies of antiviral therapy for CHC and none for CHB had details on both the length of the LB specimen and the number of CPTs.
How can adequate biopsy samples be obtained for reliable grading and staging of chronic liver disease?²⁵

A related question is whether LB can be regarded as the gold standard for the staging and grading of diffuse liver diseases when risks of biopsy, inadequate sampling, and intraobserver and interobserver errors are taken into account, not to mention the different methods of reporting. If the currently proposed minimal criteria for a liver biopsy specimen (>20-25 mm long and ≥11 CPTs) are to be used as a gold standard, then more than 1 pass using a standard percutaneous liver biopsy will be required, with more risk of complications.¹³

Thus according to Cholongitas E, et al, inadequate liver biopsy specimens (as compared to a perfect sample with the most optimal sample length and CPT criteria), have been used in studies of noninvasive markers of fibrosis.

These limitations of the liver biopsy lead to an elusive specimen which is not the “gold standard”, does accurately reflect the underlying histopathology, may not be that reliable to guide the clinicians, and finally impair the validity of nonhistological markers.

Thus, to achieve an ideal sample in routine clinical care; with a minimum requirement for a liver biopsy specimen to always be ≥ 2.5 cm longer in length, and to have 11 or more complete portal tracts could be unrealistic and dangerous for the patient on one hand, and practically very difficult on the other.
Histopathology of chronic hepatitis B and C

According to Goodman ZD, chronic necroinflammatory disease (chronic hepatitis) is a morphologic pattern seen most often in chronic viral hepatitis, but also in autoimmune hepatitis, drug reactions, and some metabolic diseases. Although the histopathologic features are similar, some notable features are more characteristic of one type than of others.

Parenterally transmitted viral hepatitis accounts for 90% of cases. Approximately 5% to 10% of cases of chronic hepatitis are autoimmune. Drug induced liver disease is a rare but well documented cause of chronic hepatitis. Metabolic diseases, such as Wilson’s disease, alpha 1 antitrypsin deficiency, and haemochromatosis, are also causes of chronic hepatitis, but they are distinguished from the usual causes of chronic hepatitis by clinical features, liver biopsy and laboratory tests.

As in acute necroinflammatory disease, hepatocellular injury and inflammation are present, but in the chronic diseases, the brunt of the injury tends to be portal and periportal rather than panacinar. And the injury is accompanied by fibrosis that can progress to cirrhosis.

Chronic hepatitis regardless of the cause, is characterized by several pathologic changes that are present to a variable extent in each case. These include the following:

Hepatic necrosis, portal inflammation and periportal injury and inflammation, piecemeal necrosis, and fibrosis which may involve only the portal and periportal areas or may form septa.

4.22 Elementary lesions of chronic hepatitis

According to Desmet V, the elementary lesions that constitute the histopathologic picture of chronic hepatitis consist of: Spotty necrosis, confluent lytic necrosis, portal inflammation, interface hepatitis, fibrosis, and cirrhosis.
Thus according to Desmet V, Spotty necrosis is an older term, loosely applied to apoptosis and necrosis of isolated hepatocytes. A more appropriate morphologic term would be, “focal necroinflammation”, recognizable as a small cluster of mononuclear cells (lymphocytes, possibly with histiocytes). Spotty necrosis in chronic hepatitis looks the same as in acute hepatitis.

Confluent lytic necrosis, comprises of confluent necrosis of the lytic type of the hepatic parenchyma, which results in dropout of adjacent groups of hepatocytes with denudation of the reticulin framework. As in acute hepatitis, the extent of confluent necrosis may range from focal and zonal confluent necrosis to “bridging” necrosis, and to the more wide spread involvement of the single or multiple lobes. It occurs in more severe forms of chronic hepatitis, and thus usually coincides with clinical episodes of disease exacerbation.

Portal inflammation in chronic hepatitis may be mild, moderate, or dense. It is composed of mononuclear cells, comprising mainly lymphocytes, variable numbers of plasma cells, and other mononuclear cells (histiocytes and immature lymphocytes). Lymphoid aggregates or follicles with germinal centers may be present and are now considered typical, although not pathognomonic, of chronic hepatitis. Immunohistochemical studies have shown that even when the germinal centers are not apparent by light microscopy, these are true functional lymphoid follicles.

Hepatitis associated bile duct lesions, according to Goodman ZD, were first described in chronic hepatitis, but the lesions may also be found in specimens of acute hepatitis. The lesion is characterized by swelling, vacuolization, nuclear irregularity, and sometimes pseudostratification of the biliary epithelial cells. The basement membrane appears to be ruptured, and lymphocytes, occasional plasma cells, and sometimes neutrophils infiltrate the duct. The lesion looks like the “florid duct lesions” of the primary biliary cirrhosis. However, the ducts are not destroyed as they are in PBC, and features of cholestasis do not develop. Reconstructions of serial sections has demonstrated that the most frequently observed lesions are actually blind diverticula arising from injured ducts rather than ducts themselves. The ductal lesions have been seen in all forms of hepatitis, but most often in hepatitis C.
Interface hepatitis, according to Goodman ZD\textsuperscript{141}, is now the preferred term for the lesion formerly known as piecemeal necrosis\textsuperscript{150,151}. The original term was defined by an international group as “destruction of liver cells at an interface between parenchyma and connective tissue, together with a predominantly lymphocytic or plasma cell infiltrate”\textsuperscript{152}. It is now apparent that the destruction of liver cells occurs primarily through apoptosis, and because the dead hepatocytes quickly disappear from the tissue, and the mode of cell death is apoptosis not necrosis\textsuperscript{153}. In addition it is the location of the inflammatory component that permits recognition of the lesion, so that interface hepatitis is the more accurate term\textsuperscript{141}.

Interface hepatitis has traditionally been considered a key lesion in the progression and pathogenesis of chronic hepatitis, and hence the distinction between chronic persistent hepatitis and chronic active hepatitis was based on this finding. The basis for this distinction is no longer considered valid, but the degree of periportal injury (mild, moderate, or severe) is still used to grade the degree of activity\textsuperscript{141}.

Interface hepatitis may be mild (one or few foci around the portal perimeter without noticeable fibrous expansion), moderate (with periportal destruction of groups of liver cells along with fibrous septa) or severe (with wedge shaped extension of the necroinflammatory lesion and obvious fibrosis deep into the lobule)\textsuperscript{143}.

According to Goodman ZD\textsuperscript{141}, Interface hepatitis can most easily be recognized as irregularity of the limiting plate, and later its disappearance, caused by the extension of portal inflammation through the plate into the periportal parenchyma. Inflammatory cells surround and destroy injured hepatocytes (“emperipolesis”). Evidence of hepatocellular degeneration and death is characterized by either acidophilic or ballooning degeneration. As in acute hepatitis, cell death occurs principally by the process of apoptosis, which results in the formation of apoptotic or acidophilic bodies that rapidly disappear from the liver plates or sinusoids. As chronic hepatitis progresses, continuous erosion of the hepatic parenchyma takes place, with closer approximation of expanded portal areas, and small groups of hepatocytes (“hepatocyte islets”) become trapped in expanded portal zones. The necroinflammatory changes are gradually succeeded by fibrosis, often best appreciated with a Masson or collagen stain. Initially, delicate collagen fibers are laid
down in areas of periportal liver cell loss, which eventually condense into scars and thick fibrous tissue. Even after cirrhosis has developed, interface hepatitis can continue at full pace along the fibrous septa, causing further loss of hepatocytes and eventually clinical decompensation of cirrhosis. Fibrosis, according to Goodman ZD, accompanies chronic hepatitis, although the degree of fibrous tissue deposition is quite variable from patient to patient. Fibrosis is the progressive component of the disease, which ultimately leads to the architectural distortion and cirrhosis. The long term prognosis of chronic hepatitis depends on the fibrous tissue accumulation. It is thought that at least two pathways may lead to fibrosis in chronic hepatitis. Probably the most important in chronic viral hepatitis is the collagen deposition that accompanies the periportal injury of interface hepatitis, causing fibrous expansion of the portal tracts.

Intralobular fibrosis apparently results from continuous ongoing lobular necroinflammatory damage. As the disease progresses, portal-to-portal fibrous bridges are formed, filling zone 1 between adjacent acini. Central-to-portal and sometimes central-to central fibrous bridges may also form, developing from superimposed episodes of portal-central (bridging) confluent necrosis involving zone 3. In the evaluation of needle biopsy specimens, it is important to distinguish tangential cuts through enlarged portal areas, which contain preexisting bile ducts and portal vessels, from true bridging fibrosis, in which septa form through parenchyma where fibrous tissue previously did not exist. Thus it reflects on the importance of reporting a certain number of complete portal tracts in liver biopsy specimens, as an attribute of an adequate sample, in addition to biopsy size.

The scars of bridging fibrosis contain elastic fibers in addition to collagen. Like scars in any tissue, these tend to contract. Contraction of the fibrous septa together with nodular regeneration of the surviving parenchyma produces architectural distortion, and when complete nodules have formed, surrounded by fibrous septa, the result is cirrhosis.
**Cirrhosis**, according to Desmet V,\textsuperscript{143} is the end result of many chronic liver diseases. It corresponds to diffuse hepatic fibrosis with replacement of normal lobular architecture by parenchymal nodules separated by fibrous issue\textsuperscript{155}. Architectural changes are best appreciated on a reticulin stain\textsuperscript{156}.

Portal-central septa linking portal tracts and central veins are an important component of cirrhosis. Cirrhosis may be strongly suspected on clinical, laboratory, and imaging parameters, but the final diagnosis requires morphological confirmation by liver biopsy\textsuperscript{143}.

The morphologic classification of cirrhosis is based on the size of nodules\textsuperscript{155}. Cirrhosis is **micronodular** if nearly all of the nodules are less than 3 mm in diameter, and **macronodular** if greater than 3 mm in diameter. A mixed **micro-macronodular** cirrhosis comprises approximately equal numbers of nodules greater than and less than 3 mm in diameter. The size of the nodules has some use in defining the aetiology of the process, but is relative since micronodular cirrhosis may convert into a macronodular type under circumstances more favorable for parenchymal regeneration\textsuperscript{143,157}.

Nodular size is more important in determining the ease with which the diagnosis of cirrhosis can be made in needle biopsy specimens. Nodularity and septa may be readily evident in specimens from micronodular cirrhosis, whereas more subtle criteria have to be relied on in macronodular cirrhosis.

According to Desmet V, helpful criteria in this respect include: fragmentation of the biopsy specimen (especially with thin tissue cylinders provided by small aspiration type needles such as Menghini needle); thin layers of connective tissue adhering to rounded edges of nodular biopsy fragments; abnormal orientation of reticulin fibers resulting from different rates of parenchymal growth in different areas; abnormal spacing of portal tracts and central veins, and excess numbers of draining veins in relation to the number of portal tracts; presence of minute and poorly formed portal tracts (mini portal tracts); hepatocellular features of regeneration – double plates over widespread areas; different appearance of hepatocytes in adjacent areas; liver cell dysplasia of the large cell and small cell type\textsuperscript{143}.

Besides, confirming the presence or absence of distorted architecture, and stage of development, the liver biopsy can help diagnose the aetiology of the liver disease, in
many instances with the use of some additional stains and attention to a number of features 143.

Before the architecture is entirely obliterated, parts of the tissue are nodular while adjacent areas maintain an acinar and viable structure, a state that can be regarded as “incomplete cirrhosis” 141. It is the most difficult anatomic type to recognize 143.

This is best characterized by irregular nodularity, slender septa, some of which end blindly, mini-portal tracts, excess efferent veins, and sinusoidal dilatation. There is evidence of parenchymal hyperplasia with corresponding compression of reticulin fibers in adjacent areas. Inflammation and necrosis are generally absent or minimal 158. The diagnosis is more easy in surgical than needle specimens 143.

The ease with which cirrhosis is diagnosed further depends on multiple factors; the type of biopsy procedure (surgical vs needle), the rout of the procedure (surgical, percutaneous, and transjugular), the type of needle used (Menghini, Trucut, or other), the adequacy of the biopsy specimen, and the quality of applied stains, and the histopathological reporting. These attributes have been discussed at length previously. In some instances, the histopathologist can only hint at the possibility of cirrhosis. A further complicated factor is the process of change of the fibrous tissue deposition. Cirrhosis does not appear overnight, but may take months or years to develop 143, a one time liver biopsy specimen may not capture the dynamic nature of the process.

4.23 Histopathology of hepatitis B virus

According to Goodman ZD, 141 hepatitis B virus can be diagnosed histologically and distinguished from chronic hepatitis of other causes by demonstration of the virus in tissue. Histochemical stains such as orcein or Victoria blue, or the more sensitive immunostains can demonstrate HBsAg in 80% or more of cases of chronic hepatitis B 141.

Cells containing large quantities of HBsAg have cytoplasm with a uniform, finely granular appearance. These ground-glass cells are scattered randomly throughout the liver, often in clusters. The number of ground glass cells tends to be inversely related to the activity of hepatitis. The greatest number of cells is found in livers with the least
active disease, whereas livers with the most activity tend to have the fewest ground glass cells\textsuperscript{141}.

In acute hepatitis B, the immune response eliminates antigen containing cells, and the results of immunostaining are entirely negative. Conversely, the presence of staining surface antigen proves a chronic rather an acute infection, even when severe hepatocellular injury is present\textsuperscript{141}.

Hepatitis B core antigen (HBcAg) can usually also be demonstrated by immunohistochemistry in nuclei and sometimes cytoplasm in cases of chronic disease. The presence of core antigen reflects active viral replication, so the amount of core is generally directly proportional to the activity of hepatitis. Patients with recent acute exacerbations have most core, with HBcAg often present in hepatocyte cytoplasm and numerous nuclei. Increased cytoplasmic core antigen has also been found in strains of virus with precore mutations associated with more severe disease\textsuperscript{141,159}.

4.24 Histopathology of hepatitis C virus

According to Goodman ZD,\textsuperscript{141} hepatitis C virus cannot reliably be demonstrated in routinely processed tissue at the present time. However, certain histologic features are characteristic, although not pathognomonic of hepatitis C\textsuperscript{149}, and there presence should prompt a serologic evaluation if it has already not been carried out\textsuperscript{141}. The overall pattern is such distinctive that entire scoring systems have been developed on the histopathologic changes associated with HCV, such as the Metavir system.

Chronic hepatitis C tends to be associated with more intense chronic portal inflammation than other types of chronic hepatitis, often with lymphoid aggregates and sometimes follicles with germinal centers. The tendency to steatosis is also greater than in other types. Approximately 50\% of biopsy specimens have some fat, and in about 10\%, the amount may be considerable. Patients infected with genotype 3 tend to have even more steatosis, and it has been suggested that the increased fat may have a cytopathic and fibrogenic effect in such cases\textsuperscript{160}. Hepatitis associated bile duct lesions may be present in acute or chronic hepatitis of any cause, but they are most frequent in hepatitis C. Severe degrees of bile duct injury can be seen in about 10\% to 15\% of
specimens from patients with chronic hepatitis C lesser degrees of duct irregularity and lymphocytic infiltration are found more often\textsuperscript{141}.

4.25 Grading and staging of chronic hepatitis

The stage of any disease is how far it has progressed in terms of its natural history, with the end stage being organ failure or death. The grade of a disease is meant to reflect how quickly it is progressing to the end stage\textsuperscript{141}. Between these, there are caveats, as discussed below:

The end stage of chronic hepatitis is cirrhosis with clinical decompensation; fibrosis or cirrhosis is less severe in earlier stages. The grade is considered to be the degree of inflammation and hepatocellular injury, which are thought to lead to the fibrosis. Of lately, it has been shown that it is the degree of development of fibrosis, which is more important of a measure of how quickly it is progressing to advanced stages than the degree of inflammation\textsuperscript{25}.

4.26 History

According to Desmet V et al,\textsuperscript{143} the introduction of needle biopsy of liver has greatly contributed to the recognition of non cirrhotic forms of chronic inflammatory liver disease. After World War II, chronic sequelae of epidemic hepatitis were described in several parts of the world\textsuperscript{161}.

By the 1960’s, chronic hepatitis was presumed to be a sequel of viral hepatitis, although a causative agent had not been identified. A simple classification of chronic hepatitis based on histology, was proposed by a group of hepatologists and pathologists in 1968\textsuperscript{162}.

According to Desmet V\textsuperscript{143}, at that time immunosuppression was the standard treatment, to be reserved for the more “active” forms of the disease, the classification distinguished between a milder form (chronic persistent hepatitis - CPH) with a low degree of necroinflammatory activity, and a more severe variant (chronic aggressive or active hepatitis – CAH) featuring higher degrees of necroinflammatory lesions; a term implying disease highly likely to progress to cirrhosis; was a form of grading.
This classification of chronic hepatitis became widely accepted and was used worldwide for nearly 40 years. Although it was emphasized that CPH and CAH were not to be considered as distinct disease entities, but as variations in disease severity of a single morphological pattern, this notion was not adhered to\textsuperscript{143}.

Since 1968, remarkable progress has been achieved in the elucidation of the multiple etiologies of chronic hepatitis, with the discoveries of HBV, HCV and HDV\textsuperscript{163}.

Piecemeal necrosis, a very important diagnostic criterion in CAH, but (incorrectly) thought by some to be pathognomonic of and synonymous with chronic hepatitis, was also observed in chronic biliary diseases such as primary biliary cirrhosis and primary sclerosing cholangitis\textsuperscript{143}.

Investigations into the natural course of the disease in chronic viral hepatitis B revealed successive phases of viral replication, elimination, and integration, associated successively with lesser, higher, and again decreasing degrees of necroinflammatory disease activity\textsuperscript{164,165}. This implied successive occurrence of CPH and CAH variants in individual patients, confusing those who considered CPH and CAH to be distinct disease entities. A change of view on chronic hepatitis was indicated\textsuperscript{166}. Finally, and most importantly, the development of more efficient treatment for viral types of chronic hepatitis (interferon and other antiviral agents) necessitated a revision of chronic hepatitis classification\textsuperscript{167-169}.

Thus advances in our understanding into the aetiologies of the diseases, the discovery of some hepatotropic viruses, and their natural history, and refinements at histopathological reporting, novel drugs of treatment, have introduced a myriad new ideas, which have fundamentally changed our concepts, and rendered this classification obsolete\textsuperscript{141}.

According to Desmet V\textsuperscript{143}, there appears to be general agreement to restrict the term “chronic hepatitis” to its original meaning: a clinical and pathologic syndrome with several causes and characterized by varying degrees of hepatocellular necrosis and inflammation. However, for lack of a better definition of chronicity, chronic hepatitis is still defined (as in 1968) as a continuous disease process without improvement for at least 6 months\textsuperscript{143,170}.
Several methods of classification are currently in use to express the grade and stage of chronic hepatitis. These can be categorized as: simple verbal descriptions, relatively simple numeric grades and stages that correspond to the verbal descriptions, and more complicated systems for numerically scoring the histologic features that correspond to the grade and stage.

Many histologic classification systems have been proposed to provide a uniform standard that can be used to compare histologic findings in clinical trials. Although some of these classifications are qualitative, quantitative systems are most frequently used in clinical trials since they are amenable to statistical analysis. In 1994 two proposals for a new classification of chronic hepatitis were published.

However, the most common quantitative system that has been used since 1981, in the assessment of chronic viral hepatitis is the Knodell score, and its modification by Ishak. In addition, another semiquantitative system, the Metavir score, has been increasingly used for chronic hepatitis C. Other scoring systems also widely used are: a simple scoring system proposed by Scheuer, and its modification by Batts and Ludwig.

Each method has its advantages and disadvantages, and the system used should be appropriate for the task at hand. In general, more complex systems can provide more information than simple ones, but they are less reproducible.

The numeric scores generated by these systems are very useful in investigational studies that involve large number of patients and require statistical analysis. They are a very good way to show differences in the histologic response between cohorts of patients receiving different forms of therapy, and they have been used successfully in many large clinical trials. However studies have shown fairly poor reproducibility of these scores when applied to individual biopsy specimens, both between different pathologists and for the same pathologist at different times.

4.27 Knodell score

According to Bonis PA, the Knodell score, also known as the histologic activity index (HAI), is composed of the summation of four individual scores representing periportal and/or bridging necrosis, intralobular degeneration and focal...
necrosis, portal inflammation, and fibrosis; the score ranges from 0 to 22. Several modifications of the HAI have also appeared, which were designed, in part, to address histologic features specific to the disease under study. One modification (referred to as the Ishak score) has six stages of fibrosis, permitting more detailed evaluation of changes in fibrosis compared with the standard Knodell fibrosis score, which has only three stages.

4.28 Limitations of the Knodell score

According to Bonis PA, the changes in the HAI are sometimes interpreted inappropriately: The standard deviation in HAI scoring among six individual observers in the original description of the Knodell score was 2.4. Thus, variation in the HAI score by less than 2.4 does not necessarily represent a true difference in the histologic pattern. This observation is not always adhered to in clinical trials and studies, in which changes of one point or more have sometimes been considered to be clinically or statistically significant. A difference of one grade has one meaning, but a difference of one stage has vastly great diagnostic and prognostic importance.

In the original description of the Knodell score, the inter- and intra-observer reliability was relatively good. However, the Knodell score was originally validated on only five patients (with a total of 14 biopsies), one of whom had hepatitis B and four of whom were presumed to have non-A, non-B hepatitis, which is most likely to have been hepatitis C.

The Knodell score is frequently used in drug trials in chronic hepatitis, particularly hepatitis C, as well as natural history studies. A decrease in the Knodell score is considered to represent histologic amelioration, or less progression to advanced fibrosis. No histologic feature represented in the Knodell score can predict the response to interferon.

The reliability of the HAI score has not been well-established in specific forms of liver disease. Reliability refers to the extent to which repeated measurements of a relatively stable phenomenon fall closely to each other.
A subsequent study evaluated the Knodell score among ten pathologists using a cohort of 30 liver biopsy specimens from patients who had documented hepatitis C. Interobserver correlation for three inflammatory components of the Knodell score was relatively poor (with kappa coefficients ranging from 0.25 to 0.46 [a perfect coefficient being 1.00]). The interobserver correlation for the total HAI was also relatively poor ranging from 0.48 to 0.57. Only the fibrosis score had good reliability with a kappa coefficient of approximately 0.80. Similar results were found for intraobserver reliability.

The HAI is weighted toward periportal necrosis and bridging necrosis. However, this weighting may have limitations. First, the Knodell score is relatively insensitive to changes in fibrosis, which is more important because it is fibrosis, and not inflammation per se, which leads to many of the sequelae of chronic liver disease. Furthermore, patients may have the same Knodell score despite having markedly different degrees of fibrosis.

Second, the individual components of the Knodell score have not been well-validated in the context of the natural history of viral hepatitis following treatment. Prior to treatment, specific components of liver histology may have different impact on disease prognosis. Thus, assessment of improvement following treatment should probably also focus on this inflammatory component of liver histology, and fibrosis (rather than lobular or portal inflammation only). This is particularly relevant in studies assessing the efficacy of long-term interferon therapy in patients with hepatitis C who did not respond to interferon or interferon in combination with ribavirin.

Histologic changes on serial liver biopsies are being used as a surrogate endpoint for determining the efficacy of treatment. Although the Knodell Score can be used as a categorical variable in statistical analysis, it is unclear what degree of change constitutes a clinically meaningful histologic response. Changes in the mean fibrosis scores in studies are used as end points, but it is not that clearly discernable in individual patients. The Knodell score does not account for features specific to different types of viral hepatitis. As an example, the HAI does not account for lymphoid aggregates, bile duct injury, and macrovesicular fat that are often present in chronic hepatitis C.
Table 11. KNODELL Histological Activity Index\textsuperscript{a}

<table>
<thead>
<tr>
<th>I. Periportal \pm bridging necrosis</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Mild piecemeal necrosis</td>
<td>1</td>
</tr>
<tr>
<td>Moderate piecemeal necrosis (involves less than 50 percent of the circumference of most portal tracts)</td>
<td>3</td>
</tr>
<tr>
<td>Marked piecemeal necrosis (involves more than 50 percent of most portal tracts)</td>
<td>4</td>
</tr>
<tr>
<td>Moderate piecemeal necrosis plus bridging necrosis\textsuperscript{b}</td>
<td>5</td>
</tr>
<tr>
<td>Marked piecemeal necrosis plus bridging necrosis\textsuperscript{b}</td>
<td>6</td>
</tr>
<tr>
<td>Multilobular necrosis\textsuperscript{c}</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Intralobular degeneration and focal necrosis\textsuperscript{d}</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Mild (acidophilic bodies, ballooning degeneration and/or scattered foci of hepatocellular necrosis in &lt; 1/3 of lobules or nodules)</td>
<td>1</td>
</tr>
<tr>
<td>Moderate (involvement of 1/3 to 2/3 of lobules or nodules)</td>
<td>3</td>
</tr>
<tr>
<td>Marked (involvement of &gt;2/3 of lobules or nodules)</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>III. Portal inflammation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No portal inflammation</td>
<td>0</td>
</tr>
<tr>
<td>Mild (sprinkling of inflammatory cells in &lt;1/3 of portal tracts)</td>
<td>1</td>
</tr>
<tr>
<td>Moderate (increased inflammatory cells in 1/3 to 2/3 of portal tracts)</td>
<td>3</td>
</tr>
</tbody>
</table>
Marked (dense packing of inflammatory cells in >2/3 of portal tracts) 4

IV. Fibrosis

No fibrosis 0
Fibrous portal expansion 1
Bridging fibrosis (portal-portal or portal-central linkage) 3
Cirrhosis\(^e\) 4

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

Table 12. Modified Histological Activity Index - The Ishak score

Grading: Necroinflammatory scores

<table>
<thead>
<tr>
<th>A. Periportal or periseptal interface hepatitis (piecemeal necrosis)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Mild (focal, few portal areas)</td>
<td>1</td>
</tr>
<tr>
<td>Mild/moderate (focal, most portal areas)</td>
<td>2</td>
</tr>
<tr>
<td>Moderate (continuous around &lt;50% of tracts or septa)</td>
<td>3</td>
</tr>
<tr>
<td>Severe (continuous around &gt;50% of tracts or septa)</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Confluent necrosis</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Focal confluent necrosis</td>
<td>1</td>
</tr>
<tr>
<td>Zone 3 necrosis in some areas</td>
<td>2</td>
</tr>
<tr>
<td>Zone 3 necrosis in most areas</td>
<td>3</td>
</tr>
<tr>
<td>Zone 3 necrosis + occasional portal-central (P-C)</td>
<td>4</td>
</tr>
<tr>
<td>Bridging</td>
<td></td>
</tr>
<tr>
<td>Zone 3 necrosis + multiple P-C bridging</td>
<td>5</td>
</tr>
<tr>
<td>Panacinlar or multiacinlar necrosis</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Focal (spotty) lytic necrosis, apoptosis and focal inflammation*</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
</tbody>
</table>
One focus or less per 10 x objective 1
One to four foci per 10 x objective 2
Five to ten foci per 10 x objective 3
More than ten foci per 10 x 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

Maximum possible score for grading 18

Modified Histological Activity Index Staging:
Architectural changes, fibrosis and cirrhosis
Change

No fibrosis 0
Fibrous expansion of some portal areas, with or without short fibrous septa 1
Fibrous expansion of most portal areas, with or without short fibrous septa 2
Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging 3
Fibrous expansion of portal areas with marked Bridging, portal-portal (P-P) as well as portal-central (P-C) 4
Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis)
Cirrhosis, probable or definite

Maximum possible score

For grading:
* Does not include diffuse sinusoidal infiltration by inflammatory cells

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

Immunohistochemical findings:
Information on viral antigens, lymphocyte subsets or other features, when available, should be recorded and may be semi-quantitatively expressed.
For staging:

Additional features which should be noted but not scored

Intra-acinar fibrosis, perivenular (‘chicken wire’ fibrosis)
Phlebosclerosis of terminal hepatic venules.


4.29 META VIR score

According to Bonis PA, 175 the Metavir score was developed in an attempt to address some of the problems with the Knodell score 126,131. In contrast to the Knodell score, which was designed as a generic scoring system for chronic hepatitis, the Metavir score was specifically designed and validated for patients with hepatitis C.

The Metavir score is a semiquantitative classifications system consisting of an activity and a fibrosis score: The fibrosis score is assessed on a five point scale (0 = no fibrosis, 1 = portal fibrosis without septa, 2 = few septa, 3 = numerous septa without cirrhosis, 4 = cirrhosis). Compared to the Knodell fibrosis score (which has only four levels), the Metavir score permits recognition of subtler variation in the degree of fibrosis. The activity score was graded according to the intensity of necroinflammatory lesions (A0 = no activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity).
Table 13. METAVIR SCORE

1. **Focal lobular necrosis**
   - Less than one necroinflammatory foci per lobule 0
   - At least one necroinflammatory foci per lobule 1
   - Several necroinflammatory foci per lobule or confluent or bridging necrosis 2

2. **Portal Inflammation**
   - Absent 0
   - Presence of mononuclear aggregates in some portal tracts 1
   - Mononuclear aggregates in all portal tracts 2
   - Large and dense mononuclear aggregates in all portal tracts 3

3. **Piecemeal necrosis**
   - Absent 0
   - Focal alteration of the periportal plate in some portal tracts 1
   - Diffuse alteration of the periportal plate in some portal tracts or focal lesions around all portal tracts 2
   - Diffuse alteration of the periportal plate in all portal tracts 3

4. **Bridging necrosis**
   - Absent 0
   - Present 1

<table>
<thead>
<tr>
<th>LN=0</th>
<th>A=0</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN=0</td>
<td>LN=1</td>
</tr>
<tr>
<td></td>
<td>LN=2</td>
</tr>
</tbody>
</table>
Algorithm for the evaluation of histological activity.

PMN, Piecemeal necrosis: 0, none; 1, mild; 2, moderate; 3, severe
LN, Lobular necrosis; 0, no or mild; 1, moderate; 2, severe
A, Histological activity; 0, none; 1, mild; 2, moderate; 3, severe

F 0= No fibrosis
F 1= Portal fibrosis without septa
F 2= Portal fibrosis with rare septa
F 3= Numerous septa without cirrhosis
F 4= Cirrhosis

The proposed algorithm provides an easy means of scoring the activity.
Adapted from\textsuperscript{126}.
The inter-and intraobserver reliability of the activity and fibrosis score of the Metavir system are similar to the Knodell score. In one study, the kappa coefficients of the Metavir activity score and the HAI, and the Metavir fibrosis score and the Knodell fibrosis score were found to be similar (approximately 0.5 and 0.8, respectively)\textsuperscript{131}. A subsequent study found that the interobserver agreement of the Metavir score depends highly upon the experience of the hepatopathologist\textsuperscript{185}. Agreement was influenced more heavily by the interpreter's experience compared with features of the specimen itself such as its length\textsuperscript{175}.

On the other hand, a separate study evaluating the fibrosis and activity scores in specimens of various length suggested that the length of the biopsy was also important\textsuperscript{170}. The kappa coefficients for fibrosis were 0.75, 0.85, and 0.92 comparing specimens of 5, 10, and 15 mm respectively (considering the fibrosis score of a 20 mm specimen as the reference standard). The corresponding figures for the activity scores were 0.73, 0.81, and 0.77, respectively. The authors concluded that a specimen length of at least 10 mm usually reflects the fibrosis and activity scores reliably. Another study, however, suggested that a length of at least 25 mm is necessary to evaluate fibrosis accurately with a semiquantitative score. Sampling variability becomes a major limitation when using more accurate methods such as automated image analysis\textsuperscript{25}.

Thus, the main advantage of the Metavir score for hepatitis C is its relative simplicity, its focus on necroinflammatory lesions, and its increased sensitivity in the fibrosis score due to the addition of one extra fibrosis level. However, many of the limitations of the Knodell score discussed above also apply to the Metavir score. In particular, the fibrosis stages of the Metavir score have not been well-correlated to the natural history of hepatitis C\textsuperscript{175}. Thus, earlier on, it was unclear whether individuals progress from early to late stages at a constant, linear rate, although this hypothesis has been proposed\textsuperscript{169}. But in subsequent studies it has been shown that the rates of progression of fibrosis were not normally distributed and greatly different estimated rates of progression were found in different patient groups\textsuperscript{186}.
4.30 Guidelines by International Association for the Study of the Liver

For routine diagnosis and patient management, one authority \(^{141}\), has recommended a simple system of grading and staging as proposed by a panel of experts convened by the International Association for the Study of Liver IASL in 1994 \(^{170}\).

Chronic hepatitis is graded according to whether the degree of activity is mild, moderate, or marked. Although this concept seems rather simple, it is essentially subjective and even this is not highly reproducible between pathologists or even the same pathologist \(^{131}\).

The principal features used to determine the grade are the degree of periportal interface hepatitis (piecemeal necrosis) and spotty parenchymal injury. The staging of chronic hepatitis is based on an assessment of the degree of fibrosis and a Masson trichrome stain is required for a proper evaluation. The stage of disease progresses as an absence of fibrosis incomplete cirrhosis, and finally to established cirrhosis \(^{141}\).

Following the recommendations of IASL panel \(^{170}\), the diagnostic line of pathology report should indicate the cause of chronic hepatitis if known, the grade, and the stage. Thus the report may read, “chronic hepatitis C with mild activity and portal fibrosis” or “chronic hepatitis B with moderate activity and extensive bridging fibrosis” or “chronic autoimmune hepatitis with marked activity and established cirrhosis” \(^{141}\).

According to the source cited above \(^{141}\), the follow up biopsy specimen of a patient who is being treated for chronic hepatitis should be evaluated in the context of previous specimens. Biopsies are performed to determine whether the activity of the patient’s liver disease has improved or the fibrosis has progressed. The only meaningful evaluation is one in which the initial specimen is compared with a follow up specimen. A comparison of pathology reports or numeric scores generated at different times, according to the source, can only lead to confusion and incorrect conclusions about the course of a patient’s disease \(^{141}\).
Table 14. Scheuer Scoring System for necroinflammatory activity and fibrosis

<table>
<thead>
<tr>
<th>Grade</th>
<th>Portal/periportal Activity</th>
<th>Lobular activity</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal or minimal</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Portal Inflammation (CPH)</td>
<td>Inflammation but no necrosis</td>
<td>Enlarged fibrotic portal tracts</td>
</tr>
<tr>
<td>2</td>
<td>Mild piecemeal necrosis</td>
<td>Focal necrosis or acidophil bodies</td>
<td>Periportal or portal -portal septa but intact architecture</td>
</tr>
<tr>
<td></td>
<td>(mild CAH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Moderate piecemeal necrosis</td>
<td>Severe focal cell damage</td>
<td>Fibrosis with architectural distortion but no cirrhosis</td>
</tr>
<tr>
<td></td>
<td>(moderate CAH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Severe piecemeal necrosis</td>
<td>Damage includes bridging necrosis</td>
<td>Probable or definite cirrhosis</td>
</tr>
<tr>
<td></td>
<td>(severe CAH)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from\textsuperscript{169}:
### Table 15. Batts and Ludwig system for Grading and staging chronic hepatitis

#### Grade

<table>
<thead>
<tr>
<th>Description</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic hepatitis, minimal</td>
<td>Grade 1</td>
</tr>
<tr>
<td>Chronic hepatitis, mild</td>
<td>Grade 2</td>
</tr>
<tr>
<td>Chronic hepatitis, moderate</td>
<td>Grade 3</td>
</tr>
<tr>
<td>Chronic hepatitis, marked</td>
<td>Grade 4</td>
</tr>
</tbody>
</table>

#### Stage

<table>
<thead>
<tr>
<th>Description</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>No fibrosis</td>
<td>Stage 0</td>
</tr>
<tr>
<td>Portal fibrosis</td>
<td>Stage 1</td>
</tr>
<tr>
<td>Few bridges</td>
<td>Stage 2</td>
</tr>
<tr>
<td>Many bridges</td>
<td>Stage 3</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>Stage 4</td>
</tr>
</tbody>
</table>

Adapted from\textsuperscript{174}:

# Table 16. Laennec scoring system for fibrosis

<table>
<thead>
<tr>
<th>Grade</th>
<th>Name</th>
<th>Septa (thickness &amp; number)</th>
<th>Descriptive examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No definite fibrosis</td>
<td></td>
<td>No septa or rare thin septum, may have portal expansion or mild sinusoidal fibrosis</td>
</tr>
<tr>
<td>1</td>
<td>Minimal fibrosis</td>
<td>+/-</td>
<td>Occasional thin septa. May have portal expansion or mild sinusoidal fibrosis</td>
</tr>
<tr>
<td>2</td>
<td>Mild fibrosis</td>
<td>+</td>
<td>Moderate thin septa, up to incomplete cirrhosis</td>
</tr>
<tr>
<td>3</td>
<td>Moderate fibrosis</td>
<td>++</td>
<td>Marked septation with rounded contours or visible nodules. Most septa are thin (one probable broad septum allowed)</td>
</tr>
<tr>
<td>4A</td>
<td>Cirrhosis, mild</td>
<td>+++</td>
<td>At least two broad septa, but no very broad septa and less than half of biopsy length composed of minute nodules</td>
</tr>
<tr>
<td>4B</td>
<td>Moderate cirrhosis</td>
<td>++++</td>
<td>At least one very broad septum or more than half of biopsy length composed of minute nodules (micronodular cirrhosis)</td>
</tr>
</tbody>
</table>

Adapted from\(^{187}\):

Fig 8  Chronic hepatitis with mild activity and no fibrosis, x 10 H&E
Fig. 9  Chronic hepatitis with fatty change (steatosis), moderate activity and portal fibrosis, x 10 H & E
Fig. 10  Chronic hepatitis with fatty change (steatosis), moderate activity and portal fibrosis, x 20 H & E
Fig. 11  Chronic hepatitis with moderate activity and portal fibrosis, x 10 H & E.
Fig. 12  Chronic hepatitis with moderate activity and portal fibrosis, x 20 H & E.
Fig. 13  Chronic hepatitis with moderate activity and bridging fibrosis, x 10 H & E
Fig. 14 Chronic hepatitis with moderate activity and bridging fibrosis, x 20 H & E
Fig. 15  Chronic hepatitis with moderate to marked activity and early cirrhosis, x 10 H & E
Fig. 16  Chronic hepatitis with moderate to marked activity and early cirrhosis,
   x 20 H & E
REFERENCES

Liver Biopsy


Quality of liver biopsy specimens


**Histopathology of Chronic Hepatitis B & C**


172. Working Party. Terminology of chronic hepatitis, hepatic allograft rejection, and nodular lesions of the liver; summary of recommendations developed by an


Chapter 5

NON INVASIVE EVALUATION OF LIVER FIBROSIS

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5.1 Introduction

Chronic liver diseases, CLD, are a major cause of morbidity and mortality in the present day world \(^1\text{-}^6\). Chronic infections with hepatitis B and C viruses, alcoholic and
non-alcoholic fatty liver diseases are important causes of CLD \(^{7-13}\). The major
determinant in their prognosis is the progressive accumulation of fibrosis, with distortion
of the hepatic architecture, and ultimate progression to cirrhosis and its allied complications \(^{3,8,9-21}\). In the recent years, with the great advancement in therapeutic
modalities to treat chronic liver diseases, accurate assessment of fibrosis has become of
paramount importance; to guide management decisions, predict outcome (prognosis), and
monitor disease activity in individual patients \(^{21-26}\).

Chronic hepatitis from HBV and HCV are the most common causes of cirrhosis
and hepatocellular carcinoma in the world today. Of approximately 2 billion people who
have been infected with HBV worldwide, more than 350 million, or about 5\% of the
world’s population are chronic carriers, and with an annual incidence of more than 50
million \(^{1,27}\). HBV accounts for 500,000 to 1.2 million deaths per year \(^4\).

Similarly more than 3 \% of the world population or about 170-5 million people
may be infected with HCV \(^2,3,28,29\). The prevalence of HCV is increasing and estimates of
the future burden of chronic hepatitis C predict at least a 3 fold rise in chronic liver
disease and cirrhosis by the year 2020 \(^3,30-32\).

Nonalcoholic fatty liver disease (NAFLD) affects 10-30\% of the general
population in various countries. 10\% of these people, or \sim 2-3\% of the general
population, satisfy the criteria for nonalcoholic steatohepatitis, (NASH). The prevalence
of NAFLD is also expected to rise in developed countries given the epidemic of its major
determinant, obesity \(^6,11,12,20,25,33-36\).

Studies indicate that advanced fibrosis and cirrhosis will develop in 20-40\% of
patients with chronic hepatitis B or C and in a similar proportion of patients with
nonalcoholic fatty liver disease \(^3,4,5,7,9,10,11,12,37,38\).

Therefore it is imperative for both clinicians and patients to acquire accurate
information about the degree of fibrosis to guide management decisions predict outcome
and monitor disease activity \(^{23}\).

Hepatic fibrosis is the final common pathway for a multitude of liver injuries.
Viral, immune, toxin mediated liver injuries all result in expansion of the extracellular
matrix, with the deposition of fibrous tissue, distortion of hepatic architecture and
ultimately the development of cirrhosis \(^39\).
Percutaneous liver biopsy is considered the gold standard for grading and staging the liver disease. But it has its limitations, which include: small but significant morbidity and mortality rates, sampling error, inter- and intra-observer variability, limits of the histopathological scoring systems, and the provision of a static picture of liver architecture in a dynamic disease process \(^{19,23}\).

With the expanding knowledge of fibrosis, more accurate, reproducible, and noninvasive methods of determining liver fibrosis are required. Understanding the pathogenesis of hepatic fibrosis at molecular level has led to the identification of several potential serum markers of hepatic fibrosis. Either individually or in combination, these serum markers appear capable of determining early and advanced fibrosis \(^{39}\).

Serum markers of hepatic fibrosis offer an attractive alternative to liver biopsy; as they are less invasive than biopsy, may allow dynamic calibration of fibrosis and may be more cost effective \(^{23}\).

Additionally, radiological means can also noninvasively assess liver fibrosis. Ultrasonography and cross-sectional imaging with CT, MRI though enables detailed images of the liver and surrounding structures, but resolution of hepatic parenchyma is insufficient to determine any of the earlier stages of fibrosis before the establishment of cirrhosis and portal hypertension \(^{40}\). Transient hepatic elastography is a novel technology demonstrating promise as a noninvasive means of fibrosis determination \(^{41}\).

### 5.2 Serum Markers

According to Afdhal N \(^{39}\), serum markers of hepatic fibrosis refer to the measurement of one or more molecules within blood or serum sample as markers of fibrosis in the liver \(^{42}\). There are several proposed biomarkers or combinations of biomarkers. Ideal features of serum markers are \(^{39}\):

- Liver specific
- Independent of metabolic alterations
- Easy to perform
- Minimally influenced by urinary and biliary excretion
• Reflective of fibrosis irrespective of cause
• Sensitive enough to discriminate between stages of fibrosis
• Correlate with dynamic changes in fibrogenesis or fibrosis resolution

According to Afdhal N 19, none of the available markers fulfill, these ideal criteria. None are liver specific, so there may be significant contribution from non-hepatic sources, including bone, joints, skin, and lungs. Levels of these markers are altered by changes in their clearance, metabolism, and excretion. For instance, both the liver (80%) and the kidneys (20%) clear hyaluronan and the removal from circulation is also dependent upon binding to specific receptors by hepatic endothelial cells 43. Increased hyaluronan levels occur in the post prandial state, presumably as a result of competition for these receptors 44.

Their relationship to the total matrix content of the liver and to the activity of fibrogenesis or degradation is usually mixed. Indeed, in the absence of a golden standard of matrix turnover, it is not possible to assess the relationship of the levels of these markers to ongoing matrix remodeling and new matrix deposition or removal. Thus from a practical and research standpoint, it would be ideal to have marker(s) that can (a) noninvasively stage the degree of liver fibrosis, (b) reflect the rate of matrix deposition or removal, both to monitor the impact of therapies and to give prognostic information. It is unlikely that any single marker can meet the acid test, and can fulfill both of these ideal criteria 19.

Proposed serum markers of hepatic fibrosis can be categorized broadly as either direct or indirect. Indirect markers reflect alterations in hepatic function but do not directly reflect extracellular matrix (ECM) metabolism, for instance platelet count, coagulation studies, and assessment of liver transaminases. Direct markers of fibrosis reflect ECM turnover. Their discovery has been linked directly to advances in understanding of the molecular mechanisms of hepatic fibrosis. Serum assays for products of matrix synthesis or degradation and the enzymes involved in these processes have been investigated as markers of liver fibrosis in several studies 45-53. Combinations of these markers, both direct and indirect, are emerging as a promising alternative to routine liver biopsy for many patients with liver disease 54-57.
5.3 Indirect Markers

According to Afdhal N\textsuperscript{39}, various indirect markers of liver fibrosis have been used in clinical practice for many years, such as on physical examination, hepatomegaly and splenomegaly. Patients who have elevated AST, ALT, coagulopathy, hypersplenism with thrombocytopenia invariably have cirrhosis and portal hypertension. Initial efforts attempted to correlate these routine investigations with stage of hepatic fibrosis \textsuperscript{39}. For example the AST/ALT ratio\textsuperscript{58}, platelet count \textsuperscript{59}, and the prothrombin index \textsuperscript{60}. It should be reiterated that the indirect markers reflect the disturbance of hepatic function or structure, rather than the deposition or the removal of ECM\textsuperscript{19}.

One of the main limitations to the clinical use of direct markers of liver fibrosis is that they are not routinely available in all hospital settings. While direct markers of liver fibrosis have shown promise in detecting liver fibrosis, the indirect markers satisfy the request for a simple and easy to perform marker with diagnostic accuracy for detecting liver fibrosis that is equal or better than direct markers.

5.4 AST/ALT Ratio

According to Afdhal N\textsuperscript{39}, Park and colleagues compared the AST/ALT ratio between 30 known cirrhotics and 123 controls without cirrhosis and reported a ratio greater than or equal to 1 had a 95.9\% specificity and 73.7\% positive predictive value in distinguishing patients who had cirrhosis from those who did not have cirrhosis, with 46.7\% sensitivity and 88.1\% negative predictive value. It appeared that though relatively insensitive, an AST/ALT greater than or equal to 1 is highly specific but not diagnostic for the presence of cirrhosis in patients who have chronic HCV infection\textsuperscript{61}.

In a cohort of patients who had hepatitis C, Assy reported a moderate correlation between AST levels and the presence of advanced fibrosis\textsuperscript{62}. A study of 252 patients who had hepatitis C evaluated the diagnostic and prognostic value of the AST/ALT ratio\textsuperscript{63}. Diagnostically, the ratio performed well; an AST/ALT ratio of greater than 1 was strongly suggestive of cirrhosis with sensitivity of 78\% and specificity 97\%. Combined use of the AST/ALT ratio with a platelet count of less than $130 \times 10^9/l$ increased the
diagnostic accuracy of the test with positive and negative predictive values of 97% and 86% respectively. Furthermore, a progressive increase in AST/ALT ratio was observed in patients with more advanced liver disease as determined by Child Pugh and MELD scores as well as by lidocaine metabolism (MEGX test).

The relative increase in AST is probably related to both reduced clearance of AST by hepatic sinusoidal cells as well as to mitochondrial dysfunction.

The value of this ratio is greatest for the noninvasive diagnosis of cirrhosis, where a ratio of >1.0 is suggestive of this diagnosis. This has been shown for both viral disease and nonalcoholic fatty liver disease. The efficacy of this ratio, is however, not relevant to all forms of the liver disease, in particular alcoholic liver disease (associated with elevated AST) or conditions associated with predominantly with hepatic inflammation (autoimmune hepatitis).

The prognostic value of AST/ALT ratio was assessed in subset of 63 cirrhotic patients who were followed for at least one year. In this subgroup, an AST/ALT ratio greater than 1.16 was found to predict mortality and performed similarly to the Child Pugh and MELD scores. It should be noted that this study included a significant proportion of patients with clinically diagnosed cirrhosis and hence it would be expected that the diagnostic accuracy of this ratio for the detection of asymptomatic cirrhosis and earlier stages of fibrosis would be lower.

5.5 PGA Index

According to Afdhal N, PGA index was the original index of hepatic fibrosis described in 1991. It was devised as a simple biological index for detecting alcoholic liver disease in drinkers. It combines the measurement of the prothrombin index, gamma glutamyl transferase (γGT) and apolipoprotein A1 (PGA). It has been validated in patients with various chronic liver diseases, in particular alcohol (hence the use of γGT). The diagnostic accuracy of the PGA score for detecting cirrhosis is reported between 66% and 72%. This was subsequently modified to the PGAA index by the addition of α2-macroglobulin, which resulted in some improvement in its performance.

Oberti, in a study of 243 patients with chronic viral or alcoholic liver disease, assessed the utility of a large number of clinical and ultrasonographic parameters as well
as indirect and direct markers of liver fibrosis to diagnose cirrhosis. Of the clinical features of cirrhosis, a firm liver with a thin lower edge had the highest diagnostic accuracy of 83%. Of the indirect markers of fibrosis, the prothrombin index had the highest diagnostic accuracy 86%, while the performance of the PGA and PGAA scores were somewhat less good at 78.5% and 80%, respectively.

In this study, of all the specific markers of fibrosis being assessed, including laminin, procollagen III N-peptide (PIIINP), hyaluronan, and TGF-β 1, only hyaluronan performed as well as the prothrombin index with a diagnostic accuracy of 86%. Of importance, it was noted that the aetiology of the liver disease affected the performance of some of these tests. For example analysis of the AST/ALT ratio had a diagnostic accuracy of 79% in viral liver disease, but as expected performed poorly in alcoholic liver disease, 65%. The PGA and PGAA scores performed better in alcoholic than viral liver disease, whereas, the prothrombin index and hyaluronan levels performed similarly in both disease groups.

5.6 Fibrotest

Fibrotest, is the most widely known, and the most validated of the tests of the noninvasive evaluation of liver fibrosis, with over 20 studies reported in the literature. Developed by Poynard and colleagues, several biochemical markers of liver fibrosis were assessed in 339 patients with hepatitis C. The aim of this study was to find out whether a panel of biochemical markers could reliably identify patients with clinically significant fibrosis (METAVIR F2 or greater) and thus be used to limit the need for liver biopsy in the selection of patients for treatment of hepatitis C.

Of the assessed markers, α2 macroglobulin, α2 globulin (or haptoglobin), γ globulin, apolipoprotein A 1, γ glutamyl transferase, and total bilirubin were the most informative, and were used to derive a calculated index; now known as the Fibrotest. In the study, a total of 11 markers were assessed: α2 macroglobulin, AST, ALT, γ glutamyl transferase, total bilirubin, albumin, α1 globulin, α2 globulin, β globulin, γ globulin, and apolipoprotein A1. The α2 globulins mainly consisted of α2 macroglobulin and haptoglobin. In the second period, Interleukin 10 (IL-10), tumor (transforming) growth
factor β1 (TGF β1), hepatocyte growth factor, apolipoprotein A₂, and apolipoprotein B were also assessed.

However, the most informative markers were, in the decreasing rank: α₂ macroglobulin, haptoglobin, GGT, γ globulin, total bilirubin, and apolipoprotein A₁.\(^{54}\)

The areas under the receiver operating characteristic curve, ROC, for the first year (0.836), and second year (0.870) did not differ (p=0.44). By selecting the upper and lower cut off values, the authors were able to categorize their patients into three groups: (1) those in which there was a high certainty of mild liver disease (METAVIR F0-1), (2) those with high certainty of significant fibrosis (METAVIR F2-4), (3) and a group of patients who could not be adequately characterized and in whom liver biopsy would be necessary, the indeterminate group\(^{39}\).

With the best index, a high negative predictive value (100% certainty of absence of F2, F3, or F4 fibrosis) was obtained in scores ranging from zero to 0.10 (12% of all patients), and high positive predictive value (>90% certainty of presence of F2, F3, or F4 fibrosis) for scores ranging from 0.60 to 1.00 (34% of all patients). The detection of significant fibrosis F2 or greater had a 75% sensitivity and 85% specificity. The assay performed somewhat better for the assessment of more advanced liver disease (METAVIR stages 3 and 4). Thus the correct identification of the disease as either mild or severe was made in 46% of the patients overall, hence obviating the need for liver biopsy in these patients. This included 100% negative predictive value for the exclusion of METAVIR stages F2-4 fibrosis and a greater than 90% positive predictive value\(^{54}\).

The same authors have validated this score in other hepatitis C positive cohorts including those with HIV infection\(^{73-75}\), but an independent study did not achieve the same results\(^76\).

Rossi et al\(^76\), studied 125 hepatitis C positive patients who had undergone liver biopsy. Using local assays and the original authors’ computer program for the calculation of Fibrotest score, the performance of the assay was somewhat less impressive.

The area under the receiver operating characteristic curve, ROC for significant fibrosis staging (> F2 on Metavir Index) was 0.739; which was smaller than the area under the ROC in the principal study. The negative and positive predictive values were 85% and 78%, respectively. Using these cut off values only 33 of the 125 patients would
have been saved liver biopsy. Six of these patients would have been misclassified as
having mild fibrosis, while the results of the liver biopsy demonstrated more significant
disease requiring treatment. The reason for this discrepancy in results is unclear, since the original authors
have shown that the assay and score appear to be reproducible when performed in
different laboratories. Commenting on the Rossi et al study, in another study the Fibropaca study, the authors had the opinion that the smaller area under the ROC may be partially explained by non respecting the analytical recommendations for performing the Fibrotest, the low number of patients, and the lack of information concerning biopsy sample. More importantly, Rossi et al, did not discuss the causes of failures for the Fibrotest and biopsy. Various studies have highlighted the importance of the pre-analytical and analytical steps in the validation of the values of biochemical markers for carrying out the Fibrotest.
The Fibropaca Study, an independent prospective multicenter study confirmed the diagnostic value of Fibrotest and Actitest found in the principal study and suggested that both the tests could be an alternative to liver biopsy in most patients with chronic HCV. In this study 504 patients were followed, 46% were classified as F2-F4 fibrosis and 39% as A2-A3 activity. The area under the ROC for the diagnosis of activity (A2-A3) was 0.73 (0.69-0.77), for significant fibrosis (F2-F4) was 0.79 (0.75-0.82), and for severe fibrosis (F3-F4) was 0.80 (0.76-0.83). Among the 92 patients (18%) with 2 fibrosis stages of discordance between Fibrotest and biopsy, the discordance was attributable to Fibrotest in 5.4% of patients, to biopsy in 3.8%, and undetermined in 9.1%.

In analyzing the discordance between liver biopsy and biomarkers, the main difficulty is the absence of a true gold standard of liver injury. The most frequent failures attributable to the markers were false positives due to Gilbert’s disease and inflammation and false negatives due to inflammation. The most frequent failures due to biopsy were false negatives due to small biopsy size. Among the eight cirrhotic patients well defined by radiological, endoscopic, or biochemical criteria with discordant Fibrotest and liver biopsy, five were not diagnosed by liver biopsy and three were not diagnosed by Fibrotest, underlining the absence of a gold standard in determination of liver fibrosis.
Nevertheless, both the Fibrotest and Acitest bring new insights in the diagnosis of fibrosis in patients with chronic hepatitis C. However, the identification of risk factors of Fibrotest and Actitest failures such as Gilbert’s syndrome, inflammation, and haemolysis must be considered before and after the Fibrotest-Actitest interpretation.

The combined use of Fibrotest and Fibroscan (an instrument used to detect transient hepatic elastography) among 183 patients with chronic hepatitis C demonstrated an area under the ROC curve of 0.88 for F ≥ 2, 0.95 for F ≥ 3, and 0.95 for F ≥ 4. When the Fibrotest and Fibroscan results agreed, liver biopsy examination confirmed them in 84% cases for F ≥ 2, in 95% of cases for F ≥ 3, and in 94% of cases for F ≥ 4. Therefore it is likely that a combination of serum markers and Fibroscan will complement each other and enhance accuracy of fibrosis detection.

5.7 Acti Test

Acti Test is a modification of the Fibrotest that incorporates ALT and reflects both necroinflammatory activity and liver fibrosis. According to the original study, the best logistic regression for diagnosis of clinically significant fibrosis (F2, F3, or F4) or substantial activity (A2 or A3) combined the same six markers as for fibrosis alone plus ALT (R² =0.297, p<0.0001).

Acti Test appears to demonstrate improved identification of more advanced fibrosis associated with greater histologic inflammation. Both Acti Test and Fibrotest have shown reductions among those with sustained virological response to interferon and ribavirin therapies, supporting a role in following response to therapy.

A meta-analysis of 16 publications and 1570 patients, by the same group concluded that: at a cut off of 0.31, the Fibrotest negative predictive value for excluding significant fibrosis was 91%. At a cut off of 0.36, the Acti Test negative predictive value for excluding significant necrosis was 85%. Additionally, there was no difference between the area under the ROC’s of Fibrotest/Acti Test according to the genotype or viral load. Thus the use of biochemical markers of liver fibrosis (Fibrotest), and necrosis (Acti Test) can be recommended as an alternative to liver biopsy for the assessment of liver injury in patients with chronic hepatitis C.
5.8 Steato Test

Developed by Poynard and colleagues\textsuperscript{84}, Steato Test incorporates the five components of Fibrotest ($\alpha_2$ macroglobulin, haptoglobin, apolipoprotein A\textsubscript{1}, $\gamma$ glutamyl transferase, total bilirubin) and Acti Test (ALT) plus body mass index, serum cholesterol, triglycerides, and glucose adjusted for age and gender. A cut off of 0.30 had a 90% sensitivity and a cut off of 0.72 had a 90% specificity permitting to achieve a useful predictive value, 93% NPV and 63% PPV for a steatosis prevalence of 30%. Although the PPV of the Steato test is not that high, but still is significantly higher than those of previous markers to evaluate steatosis, such as, GGT, ALT, and ultrasonography\textsuperscript{84}.

5.9 Forns index

Forns et al\textsuperscript{55}, reported an index based on four readily available variables; age, platelet count, $\gamma$ glutamyl transferase, and cholesterol levels. The study was confined to patients with hepatitis C and included both test and validation cohorts. The authors constructed a simple score system applying a constant to the obtained formula:

$$7.811 - 3.131 \ln(\text{platelet count}) + 0.781 \ln(\text{GGT}) + 3.467 \ln(\text{age}) - 0.014 \cdot (\text{cholesterol})$$

Using the test cohort of 125 patients, in a manner similar to the Imbert-Bismut study (Fibrotest)\textsuperscript{54}, the authors set thresholds for defining individuals with a high or low probability of significant fibrosis (METAVIR F2-4). The area under the ROC curve was 0.86 for the estimation group and 0.81 for the validation group\textsuperscript{55}. When these criteria were applied to the validation cohort, 51% of the population could be classified using these upper and lower cut off values. The lower cut of value had a 96% negative predictive value, whereas the upper cut off value had a positive predictive value of 66%. Hence this test was useful at excluding patients with minimal fibrosis, but was of limited value for the identification of patients with more advanced liver disease\textsuperscript{19}. In summary half of the patients with chronic hepatitis C without significant liver fibrosis can be identified with high accuracy with this panel; and liver biopsy could have been avoided in more than one third of the patients\textsuperscript{55}.

One important aspect of the index is the use of very basic clinical parameters, and the fact that the accuracy of this model compares with models based on more
sophisticated variables (such as Fibrotest). In comparing with the study by Imbert-Bismut (Fibrotest) some of the variables used do provide a high level of certainty of the presence or absence of significant fibrosis, but the usefulness may be curtailed by the fact that some of the predictive markers (α2 macroglobulin, haptoglobin, and apolipoprotein A1) are research tools not readily available in routine clinical practice in most centers.

In addition, the patient population analyzed in the Imbert-Bismut study included a high proportion of patients with advanced hepatitis C infection, as indicated by the presence of significant fibrosis in up to 40% of the subjects. In the authors’ series, significant fibrosis was present in only 25% of the subjects a figure possibly closer to the spectrum of the disease in the community at large.

Additionally, the performance of this index was compared to the Fibrotest by the authors of the later test. They found that the results of the Forns index were reproducible but performed slightly less well than Fibrotest.

Major caveats of the Forns index include concerns about the impact of lipid abnormalities in patients with hepatitis C, cholesterol altering medicines, and the reproducibility of platelet estimations.

5.10 APRI (AST to Platelet Ratio Index)

Reported by Wai and colleagues, APRI refers to the AST to platelet ratio index. In this retrospective study, a total of 270 patients were evaluated, which included training (n=192), and validation cohorts (n=78). The authors looked at a number of simple laboratory studies and their relationship to two end points, significant liver fibrosis and cirrhosis. Based on their analysis of simple laboratory measurements, they found that the APRI was the simplest and the most accurate test for the evaluation of two end points.

It is calculated as follows:

\[ \text{APRI} = \frac{\text{AST level} \div \text{ULN}}{\text{Platelet count (}10^9/l)} \times 100 \]
Platelet counts and AST levels are the most important predictors of significant fibrosis and cirrhosis. This novel index was developed to amplify the opposing effects of fibrosis on AST and platelet count.

The area under the ROC curve of APRI for predicting significant fibrosis and cirrhosis in the training set were 0.80 and 0.89, respectively. In the validation cohort the same values were 0.88 and 0.94, respectively. While the upper and lower cut off levels were selected with the aim of correctly characterizing this cohort into those with significant fibrosis and cirrhosis, the index performed reasonably well. Thus, using the optimized cut off values, significant fibrosis could be predicted accurately in 51% and cirrhosis in 81% of patients.

APRI test successfully and simply can exclude those with and without significant fibrosis and cirrhosis with negative predictive values of 86% and 98%, respectively; and corresponding positive predictive values of 88% and 57%.

Its most important aspect is the use objective and readily available laboratory values. Both platelet count and AST levels are routine tests performed in chronic hepatitis C patients in clinical practice, so no additional tests are required. The finding of decreased platelet count and increased AST level with progression of liver fibrosis has been reported in many studies.

With increasing fibrosis and worsening portal hypertension, there is increased sequestration and destruction of platelets in the enlarging spleen. In addition, studies in liver transplant patients showed that the progression of liver fibrosis is associated with decreased production of thrombopoietin by hepatocytes, and hence reduced platelet production. Progression of liver fibrosis may reduce the clearance of AST, leading to increased serum AST levels. In addition, advanced liver disease may be associated with mitochondrial injury, resulting in more marked release of AST, which is present in mitochondria and cytoplasm, relative to ALT.

One caveat of the study is that: it included a sufficient proportion of patients with significant fibrosis (47%) and cirrhosis (15%) from a tertiary care center; so the results may not be totally generalizeable to community based practice.
Though slightly inferior, nevertheless, its simplicity (APRI can be determined by the bedside with the help of a calculator), matched with performance to the more sophisticated Fibrotest and Forn’s index is a great advantage\(^\text{39}\).

### 5.11 Göteborg University Cirrhosis Index (GUCI)

In this study\(^\text{88}\), samples from 179 patients with chronic hepatitis C were analyzed using routinely available biochemical markers of liver disease, and compared with liver biopsy using the Ishak protocol. By multivariate logistic regression analysis the authors found strong association between AST, platelet count and prothrombin-INR. They developed the Göteborg University Cirrhosis Index (GUCI) according to the formula:

\[
\text{Normalized AST} \times \text{prothrombin-INR} \times 100 \\
\text{Platelet count (x 10}^{9}/l)
\]

Using a cut-off value of 1.0, the sensitivity was 80%, and the specificity 78% for the diagnosis of cirrhosis, and the negative predictive value and the positive predictive values were 97% and 31% respectively. The authors concluded that the GUCI score proved slightly superior for sensitivity, specificity, NPV, PPV, and the area under the receiver operating characteristic curve (ROC) for the prediction of cirrhosis and bridging fibrosis compared with AST to platelet ratio index (APRI)\(^\text{88}\).

### 5.12 Direct Markers of Liver Fibrogenesis

According to Afdhal N\(^\text{39}\), the extracellular matrix refers to a group of macromolecules (both soluble and insoluble) that comprise the supporting framework of the normal and fibrotic liver. The liver fibrosis results in qualitative and quantitative changes in ECM markers. Some ECM markers reflect fibrogenesis, and others reflect fibrosis regression, a dynamic evaluation of liver fibrosis reflecting ECM activity is possible.

The direct markers of liver fibrosis include a number of serum or urinary markers which have been shown to be, or are thought to be directly involved in the deposition or
removal of ECM (see table 17) 19. The potential markers of fibrosis include products of collagen synthesis or degradation, enzymes involved in matrix biosynthesis or degradation, extracellular matrix glycoproteins, proteoglycans and glycosaminoglycans 39.

None of the currently available direct markers completely fulfill the ideal criteria. In particular, none is liver specific, and all are affected by changes in their clearance, metabolism, and excretion. The serum half lives of these molecules is short lived, so levels probably reflect the activity of ECM metabolism. Since the ECM turnover is related to both new ECM deposition and removal, as well as remodeling of established ECM, serum levels probably reflect both the activity of the process as well as the total mass of ECM undergoing metabolism 19.

This is supported by at least three features. First levels of these markers are most often elevated in conditions with rapidly progressing fibrosis (e.g., severe alcoholic hepatitis or more active hepatitis) and may be high prior to the deposition of ECM 89,90. Second, levels tend to fall in response to treatment of the underlying disease process, often before to any discernible reduction in the stage of fibrosis 91,92. Third in chronic liver diseases, elevations of several, but not all of these markers correlate independently with the stage of fibrosis, rather than with either biochemical or histological features of inflammation 53,93-95.

However, in some studies serum levels of these markers correlated more strongly with the degree of histological inflammation or transaminases 96,97. The observation that markers of ECM metabolism are increased in parallel with markers of liver inflammation and necrosis may reflect the importance of these processes in upregulating fibrogenesis 19.

According to Afdhal N 19, the direct markers of liver fibrosis can be conveniently subdivided into three groups: Those reflecting (a) matrix deposition, (b) matrix removal, or (c) where the relationship to the matrix deposition or removal is unclear.

One approach to the evaluation of matrix deposition is to simultaneously measure markers of both matrix deposition and removal, with a view to define the net effect upon the fibrotic process. However, using combination of these markers has added
little diagnostic accuracy, while increasing both the expense and the complexity of interpreting results\textsuperscript{19}.

<table>
<thead>
<tr>
<th>Table 17. Direct markers of liver fibrosis\textsuperscript{19}</th>
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<tbody>
<tr>
<td><strong>Markers of matrix removal</strong></td>
</tr>
<tr>
<td>Procollagen IV C peptide</td>
</tr>
<tr>
<td>Procollagen IV N peptide (7-S collagen)</td>
</tr>
<tr>
<td>Collagen IV</td>
</tr>
<tr>
<td>Metalloproteinase MMP</td>
</tr>
<tr>
<td>Undulin</td>
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<tr>
<td>Urinary desmosine and hydroxylysylpyridinoline</td>
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<tr>
<td><strong>Markers of matrix deposition</strong></td>
</tr>
<tr>
<td>Procollagen I carboxy terminal peptide (PICP)</td>
</tr>
<tr>
<td>Procollagen III amino terminal peptide (PIIINP)</td>
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<tr>
<td>Tissue inhibitor of metalloproteinase (TIMP)</td>
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<tr>
<td>Transforming growth factor beta (TGF-(\beta))</td>
</tr>
<tr>
<td>Tenascin</td>
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<tr>
<td><strong>Uncertain</strong></td>
</tr>
<tr>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>YKL-40 (Chondrex)</td>
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<td>Laminin</td>
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Fibrosis markers can also be classified according to their molecular structure. These include: (a) the collagens; procollagen I and III, propeptides released into the serum during matrix deposition and remodeling. Type IV collagen, which is released during interstitial filament degradation, reflects matrix degradation and remodeling. Several assays for type IV collagen are available but their performance tends to be similar; (b) The glycoproteins and polysaccharides including hyaluronan, laminin, tenascin, and YKL-40, which are found in association with the basement membranes and in regions of matrix deposition. The relationship between these markers and matrix metabolism has not been clearly defined; (c) Collagenases and their inhibitors, include the metalloproteinases (MMP’s) and the tissue inhibitors of metalloproteinases (TIMP’s); (d) Cytokines involved in liver fibrosis, the best studied of these is TGF-β. Others including platelet derived growth factor and the antifibrotic cytokine IL-10, have been less well evaluated.

5.13 Hyaluronic Acid

According to Afdhal N, in evaluating single marker assays that reflect ECM concentration, the best individual test appears to be hyaluronic acid (HA), which has been validated in many clinical trials. Studies have demonstrated that serum hyaluronic acid levels correlate with the degree of hepatic fibrosis in patients who have chronic hepatitis C. HA is a high molecular weight glycosaminoglycan, which is an essential component of extracellular matrix in virtually every tissue in the body. In the liver, HA is mostly synthesized by the hepatic stellate cells and degraded by the sinusoidal endothelial cells. HA levels are increased in chronic liver diseases. In patients with chronic hepatitis C virus, HA levels increase with the development of liver fibrosis. Moreover, in patients with cirrhosis, HA levels correlate with clinical severity.

Serum hyaluronic acid can identify those without cirrhosis accurately. A level of 60 µg/l had a 99% negative predictive value for the absence of cirrhosis. It had low accuracy for diagnosing cirrhosis (30% positive predictive value).
A study by Halfon et al. focused on the diagnostic accuracy of HA alone, (instead of combination of markers) in predicting fibrosis and cirrhosis in HCV infected patients. In all 405 patients were studied. Absence of significant fibrosis, severe fibrosis, and cirrhosis can be predicted by HA levels of 16, 25, and 50 µg/l respectively (with NPV of 82%, 89%, and 100% in the same order). Presence of significant fibrosis, severe fibrosis, and cirrhosis can be predicted by HA levels of 121, 160, and 237 µg/l respectively (with PPV of 94%, 100%, and 57% in the same order). Thus serum HA is a clinically useful as a noninvasive marker of liver fibrosis and cirrhosis. It suffers from the need to limit, as much as possible, potential confounding variables such as the effects of exercise and eating.

According to Afdhal N, Oberti evaluated four specific markers of fibrosis (PIIINP, HA, Laminin, TGF-β). These specific markers of fibrosis were studied in 243 patients with viral and alcoholic liver disease. In this prospective study HA performed best with a diagnostic accuracy of 86%, whereas the performance of other specific markers of fibrosis was less impressive; PIIINP 74%, laminin 81%, and TGF-β 67%. Overall, the results of this study did not demonstrate any diagnostic advantage of the specific over the nonspecific markers of fibrosis.

Other comparative studies have also supported the superiority of HA over PIIINP for the diagnosis of cirrhosis. For example in a comparative study of 326 patients with hepatitis C, areas under the receiver operating curve (ROC) for the diagnosis of fibrosis and cirrhosis were 0.86 and 0.92 for HA, while they were 0.69 and 0.73 for PIIINP respectively. One explanation for the difference in performance may be related to the observation that PIIINP levels correlate more closely with histological and serum markers of hepatic inflammation than HA.

The greatest clinical utility of HA may be its ability to exclude patients with significant fibrosis and cirrhosis. In a study of 486 patients who entered the hepatitis C Consensus Interferon Study, an HA level of <60 mg/l was found to exclude patients with cirrhosis and significant hepatic fibrosis with predictive values of 99% and 93% respectively. Hence a low HA level may have a special role in identifying patients with early fibrosis and hence reduce the need for biopsy in this subgroup of patients.
YKL-40 (Chondrex)

YKL-40 is a novel marker of liver fibrosis. YKL-40 (chondrex, human cartilage glycoprotein-39), according to Saitou et al [106], is a recently described glycoprotein that belongs to the chitinase family [107]. YKL-40 mRNA was strongly expressed in human liver and arthritic articular cartilage [107,108] and was elevated in the synovial fluid [109], alcoholic liver disease [110], recurrent breast cancer, and colorectal cancer [106].

Although its physiologic function is not known in detail, YKL-40 is thought to contribute to tissue remodeling or degradation of the extracellular matrix in liver fibrosis [111]. YKL-40 is produced by a wide variety of cells, including: chondrocytes, synovial cells [109], activated macrophages, neutrophils, and in particular from cells located in tissues with increased remodeling/degradation or inflammation of the extracellular matrix, such as hepatic stellate cells [111]. It is growth factor for fibroblasts [112], chondrocytes and synovial cells. It is a chemo-attractant for endothelial cells [113], modulates vascular endothelial cell morphology by promoting the formation of branching tubules, indicating that YKL-40 may have a role in angiogenesis [106].

The authors [106] studied Type IV collagen, amino-terminal peptide of type III procollagen (PIIIP), hyaluronic acid (HA), YKL-40 and biochemical parameters. The authors concluded that HA and YKL-40 were more useful than other markers for assessing the fibrosis stage. In particular, YKL-40 was the most useful for monitoring the fibrosis of liver disease and for distinguishing extensive liver fibrosis from mild stage of liver fibrosis, enabling to predict severe stage of fibrosis at 80% positive predictive value. It appears that serum levels of YKL-40 represented ongoing fibrosis like HA, in addition to fibrogenesis similar to type IV collagen and PIIIP of the liver disease.

Serum YKL-40 levels is valuable for diagnosing mild stage of fibrosis (value < 186.4), severe stage of fibrosis (186.4 < value < 284.8), and F4 (284.8 < value). HA appeared to be slightly better for prediction of cirrhosis (F4) from chronic hepatitis (F0-3) than YKL-40. Additionally, after Interferon therapy, only YKL-40 values significantly decreased not only in the responder group, but also in the non-responder group [106].

Further studies have also shown that YKL-40 appears to be reduced in patients with chronic hepatitis C undergoing treatment, in conjunction with several other direct markers of hepatic fibrosis, including PIIINP, MMP-2, and TIMP-1 [114]. In a study of
YKL-40 in alcoholic liver disease suggested that it could function as an independent marker of clinical outcomes.  

5.15 Collagen  

According to Afdhal N, collagen, the major component of hepatic fibrosis, is synthesized in a precursor form known as procollagen. Enzymatic cleavage by two distinct enzymes liberates carboxy and amino terminal procollagen peptides that have proposed correlation with fibrogenesis. Procollagen type III amino terminal peptide (PIIINP) is known to be elevated in acute hepatitis, and levels reflect stage of fibrosis in chronic liver disease. PIIINP does not perform as well as HA for predicting fibrosis but does reflect inflammatory score better.  

Type IV collagen has been immunolocalized to the periportal interstitium and large fibrotic bands in alcoholic liver disease. Serum type IV collagen is increased in chronic liver diseases relative to controls. Some studies have demonstrated equivalent efficacy in fibrosis determination to that seen with HA, but results have been inconsistent.  

According to Afdhal N, Murawaki et al., assessed the diagnostic performance of two forms of type IV collagen (7s domain and central triple helix domain) in 151 patients with viral hepatitis. While the two assays performed similarly, the 7s domain achieved slightly greater diagnostic accuracy for identification of patients with cirrhosis, with positive and negative predictive values of 75% and 92% respectively. In another study from the same group, the correlation between two assays for type IV collagen and fibrosis scores were similar to HA but better than PIIINP. However, the correlation to histological inflammatory scores and ALT values were greater, suggesting that type IV collagen may be a marker of both inflammation and fibrosis.  

At this time there appears to be no clear advantage to the use of assays of type IV collagen over HA for fibrosis staging.
5.16 Matrix metalloproteinases and inhibitors

According to Afdhal N 39, the matrix metalloproteinases (MMP’s) and their inhibitors are a group of proteins involved in controlling matrix degradation. MMP’s are enzymes that are produced intracellularly and secreted in a proenzyme form that requires cleavage by cell surface mechanisms for functional activity. The action of MMP’s is counteracted by tissue inhibitors of metalloproteinases (TIMP’s) 39.

As such these proteins act to both degrade ECM and to permit new matrix deposition. However, the relationship is complex and in addition to the actions cited above; these molecules have multiple other activities including: activation of growth factors, effects on cell proliferation, and inhibition of apoptosis. Thus the association between peripheral levels and liver fibrosis is not clear 19.

The hypothesis that hepatic fibrogenesis is associated with disrupted matrix degradation stems from hepatic stellate cell research. MMP-13 is decreased in activated stellate cell culture, while TIMP activity increases, promoting accumulation of extracellular matrix. This corresponds to findings in human cirrhotic livers explants that demonstrate increased expression of TIMP’s 120,121. Therefore, it seems that imbalance between MMP’s and TIMP’s affects rate of fibrosis progression, and their estimation correlates with stage of fibrosis. Results from studies of these markers, however, have been variable and dependent upon the MMP/TIMP being assessed 51,115,122,123.

According to NA 19, Boeker et al 122, reported on the diagnostic accuracy of TIMP-1 and pro-MMP-2, the free precursor molecule of MMP-2, and their relationship to histological inflammatory scores. In this study of 78 patients with hepatitis C infection, both of these assays performed as well or better than HA for the diagnosis of cirrhosis, but only TIMP-1 showed diagnostic value for the identification of patients with earlier stages of fibrosis. MMP-2 levels become elevated only once cirrhosis has developed.

Disturbing characteristics of these assays include the observation that TIMP-1 levels correlate strongly with histological inflammatory scores and that in the noncirrhotic liver MMP-2 levels demonstrated no relationship to the stage of fibrosis. These results are similar to other studies and significantly limit their value for staging liver disease 123,124. Serum MMP-1 and MMP-3 levels have not been shown to be of significant diagnostic value 51,125.
5.17 Cytokines

Cytokines are thought to mediate hepatic fibrogenesis have also been evaluated in determining hepatic fibrogenesis in a limited number of studies with mixed results\textsuperscript{19}. TGF-\(\beta\) is the dominant stimulus for producing extracellular matrix by hepatic stellate cells. In a study of 88 patients who had chronic hepatitis C, there was a correlation between total TGF-\(\beta\) \(1\) and the degree of hepatic fibrosis\textsuperscript{126}.

In another study of 39 patients with two liver biopsies, a close correlation was noted between TGF-\(\beta\) serum levels and the rate of fibrosis progression, which may suggest the use of TGF-\(\beta\) to determine those with progressive disease suitable for antiviral therapy\textsuperscript{127}. But the performance of TGF-\(\beta\) appears to be less than the other already discussed markers\textsuperscript{54,70,126}.

According to Afdhal N\textsuperscript{19}, a few studies have evaluated the relationship between the levels of soluble adhesion molecules and liver inflammation and fibrosis\textsuperscript{128,129,130}. Increased expression of intracellular adhesion molecule-1 (ICAM-1) occurs in virus infected hepatocytes; whereas expression of vascular adhesion molecule-1 (VCAM-1) may be seen in association with progressive hepatic fibrosis\textsuperscript{128-130}. It is thought that increased VCAM-1 expression occurs in association with capillarization of the sinusoidal spaces and fibrous septa.

In a study of 52 patients with hepatitis C virus, soluble VCAM-1 was found to have excellent discriminant power for the detection of advanced fibrosis, sensitivity 100\%, and specificity 85\%. In this small study, measurement of soluble VCAM was of greater diagnostic value than PIIINP\textsuperscript{128}. Further studies are needed to confirm these results, in larger patient populations and in other disease cohorts\textsuperscript{19}.

Panels with Indirect & Direct markers of liver fibrosis

Combinations of direct markers have been tried with indirect markers to enhance the accuracy of liver fibrosis detection. Some of the assays are:
5.18 FibroSpect

McHutchison and colleagues in a retrospective cohort study evaluated the FibroSpect assay. The aim of the study was to evaluate the diagnostic accuracy of a panel of markers in chronic hepatitis C patients, develop a predictive algorithm that differentiates no/mild (METAVIR F0-F1) from moderate/severe (F2-F4) fibrosis and validate the model in external cohorts. The assay involves three parameters: hyaluronic acid, TIMP-1, and α2 macroglobulin. These three markers were selected as having the best predictive accuracy for F2-F4 fibrosis (combined AUROC = 0.831).

At an index cut off of >0.36 and prevalence for F2-F4 of 52%, results in all 696 patients indicated positive and negative predictive values of 74.3% and 75.8% with an accuracy of 75%. All patients were evaluable by the FibroSpect with no indeterminate values, and this is an advantage of the assay. Maximum sensitivity and specificity were seen at the two extreme spectrums of disease, stage 0 and stage 4. The authors concluded that the three marker panel may reliably differentiate chronic hepatitis C patients with moderate/ severe fibrosis from those with no/mild fibrosis; although accurate delineation between stages was not possible. In clinical use, the test gives a likelihood of prediction as to whether the patient has mild or advanced disease based on the score.

5.19 FibroSpect II

In a recently reported retrospective study, hyaluronic acid, YKL 40, and FibroSpect II (comprising hyaluronic acid, TIMP-1 (tissue inhibitor of metalloproteinase 1), and alpha 2 macroglobulin) were assessed with Ishak stages and digital quantification of fibrosis (DQF). Among the serum markers, hyaluronic acid was effective in discriminating Ishak stages 0-1 and Ishak stages 2-3 compared with FS-II, with area under the ROC curve of 0.76 versus 0.66 respectively. All three serum markers predicted advanced fibrosis and cirrhosis. YKL-40 had the highest false positive rates in all categories of fibrosis.
5.20 European Liver Fibrosis Group assay

The ELF group\textsuperscript{133} reported a novel assay in an international multi-center cohort of 1021 patients with hepatitis C, nonalcoholic fatty liver disease, and alcoholic liver disease. The authors aimed to develop a panel of sensitive automated immunoassays to detect matrix constituents and mediators of matrix remodeling in serum to evaluate their performance in the detection of liver fibrosis.

Serum levels of 9 surrogate markers of liver fibrosis were compared with fibrosis stage in liver biopsy specimens obtained from all 1021 subjects with chronic liver disease. Discriminant analysis of a test set of samples was used to identify an algorithm combining age, hyaluronic acid, amino-terminal propeptide of type III collagen (PIIINP), and tissue inhibitor of matrix metalloproteinase 1.

The sensitivity for the detection of Scheuer stage 3 or 4 fibrosis was 90\% at a threshold score of 0.102, and accurately detected the absence of fibrosis (negative predictive value for significant fibrosis, 92\%; area under the curve of a receiver operating characteristic plot, 0.804; standard error, 0.02; p < 0.0001; 95\% confidence interval, 0.758-0.851). The algorithm performed equally well in comparison with each of the pathologists. In contrast, the pathologists’ agreement over histological scores ranged from very good to moderate (kappa = 0.97-0.46). Thus the algorithm achieved a similar level of sensitivity and specificity when compared with the scoring of three different pathologists, providing evidence that it could be used with similar accuracy in different settings.

The authors concluding that assessment of liver fibrosis with multiple serum markers used in combination is sensitive, specific, and reproducible, suggesting they may be used in conjunction with liver biopsy to assess a range of chronic liver diseases\textsuperscript{133}.

5.21 Fibrometer

In this landmark study\textsuperscript{134}, the authors studied 383 patients with viral hepatitis, 95 patients with alcoholic liver disease in the exploratory step, and the validating population consisted of 120 patients with chronic liver disease due to HCV. The objective was to develop tests to characterize different fibrosis parameters (Fibrometer) in viral and
alcoholic liver diseases. Measurements included 51 blood markers, and Fibrotest, Fibrospect, ELFG, APRI, and Forns scores. The clinically significant fibrosis was evaluated via Metavir staging (F2-F4), and image analysis was used to determine the area of fibrosis (AOF).

In patients with chronic viral hepatitis, the area under the receiving operator characteristic (AUROC) curve for stages F2-F4 in a test termed the “Fibrometer” test, combining platelets, prothrombin index, AST, α2 macroglobulin (A2M), hyaluronate, urea, and age was 0.883 compared with 0.808 for the Fibrotest (p = 0.01), 0.820 for the Forns test (p = 0.005), and 0.794 for the APRI test (p < 10⁻⁴). The Fibrometer AUROC curve was 0.892 in the validating step in 120 patients. In patients with alcoholic liver disease the AUROC curve for stages F2-F4 in a test combining prothrombin index, A2M, hyaluronate, and age was 0.962.

The area of fibrosis was estimated in viral hepatitis by testing for hyaluronate, γ glutamyl transferase, bilirubin, platelets, and apolipoprotein A1, the adjusted R² coefficient in linear regression (\(\alpha R^2 = 0.645\)), and in alcoholic liver diseases by testing for hyaluronate, prothrombin index, A2M, and platelets (\(\alpha R^2 = 0.836\)).

The 95 patients with alcoholic CLD were significantly older and had more marked fibrosis than patients with viral CLD, something that could have influenced the statistical results.

The authors claimed that their study was original because it measures the area of fibrosis, AOF, in addition to histological staging, takes into account the cause of CLD, and includes a large number of blood variables (n = 51).

The results of blood tests for clinically significant fibrosis were better in the largest specimens, thus test results could be even better with new criteria for specimen length, \(\geq 20\) mm \(^{135,136}\). It has been suggested that the variability is greater for AOF evaluation than it is for histological staging \(^{135}\). AOF is the only quantitative morphological method of determining the amount of liver fibrosis, and it has been suggested that it is superior to histological staging \(^{95}\). For instance, there is restriction of cirrhosis to one stage (F4), whereas the amount of fibrosis in cirrhosis is four times that of the other four stages \(^{95}\).
Although there was less observer variability for histological staging when a consensual reading was performed by two experts\textsuperscript{137}, blood test results were not perfect (e.g., the diagnostic accuracy, DA, for the Fibrometer test was 83.3\% in the validation population). This might be due to preanalytical or analytical variability in blood variables or variability in the pathological reference. Studies with the Fibrotest have suggested that most errors are due to histological staging itself\textsuperscript{80,138}.

The authors commented that the plots of the blood test results and of interobserver agreement in relation to F stages\textsuperscript{137}, all had the same V-shape with a nadir at the F2 stage, suggesting that the difficulty in distinguishing F2 from F1 or F3 stages via histological staging was the main cause of misclassification by the blood tests\textsuperscript{134}.

The use of blood tests to estimate the area of fibrosis is new. Measurement of area of fibrosis via image analysis is limited to clinical research. Unlike histological staging, the AOF provides precise quantification of the extensive variations in fibrosis during cirrhosis. AOF estimation via blood test is the only statistically validated quantitative test for the noninvasive diagnosis of fibrosis. However, the aim of AOF evaluation is not to distinguish mild stages due to overlap of AOF values in these stages\textsuperscript{134}.

The authors concluded that: the pathological staging and the area of liver fibrosis can be estimated using different combinations of blood markers in viral and alcoholic liver diseases. The Fibrometer has a high diagnostic accuracy for clinically significant fibrosis; blood tests for the area of liver fibrosis provide a quantitative estimation of the amount of fibrosis, which is especially useful in cirrhosis\textsuperscript{134}.

5.22 Hepascore

In this study\textsuperscript{139}, the authors evaluated 10 biochemical biomarkers at the time of liver biopsy in 117 untreated hepatitis C patients, and further validated in 104 patients. Multivariate logistic regression and ROC curve analyses were used to create a predictive model for significant fibrosis (METAVIR F2, F3, and F4), advanced fibrosis (F3 and F4), and cirrhosis (F4).

A model, Hepascore, consisting of bilirubin, \(\gamma\) glutamyl transferase, hyaluronic acid, \(\alpha2\) macroglobulin, age and sex produced areas under the ROC curves of 0.85, 0.96 and 0.94 for significant fibrosis, advanced fibrosis, and cirrhosis, respectively. In the
training set, the model was 92% specific and 67% sensitive for significant fibrosis, 81% specific and 95% sensitive for advanced fibrosis, and 84% specific and 71% sensitive for cirrhosis.

Thus a model consisting of four serum markers plus age and sex provides clinically useful information regarding different fibrosis stages among hepatitis C patients.

### 5.23 SHASTA Index

Developed by Afdhal N and colleagues\(^{140}\), the SHASTA index consists of serum hyaluronic acid, AST, and albumin. It was evaluated in a cohort of 95 patients with HIV/HCV coinfection. As with other biomarker assays, optimal results were noted in the extreme categories. Using a cut off of 0.8 resulted in a specificity of 100% and a positive predictive value of 100%, but this applied to less than 5% of patients. At the other end of the spectrum, a cutoff of less than 0.30 was associated with a sensitivity of more than 88% and a negative predictive value of more than 94%. Overall 42% of patients could be correctly classified at either extreme, but 58% would not be classifiable with scores between 0.3 and 0.8. The SHASTA index in HIV/HCV has similar accuracy to the Fibrotest and in this study performed significantly better than the APRI test.

### 5.24 APRICOT Clinical Investigators Assay

A retrospective analysis of liver biopsies was performed in 832 patients with HIV/HCV co-infection, who were randomly assigned to training (n = 555), and validation (n = 277) sets.

The authors derived a simple index (FIB-4)\(^{141}\):

\[
\text{Age (yr) x AST [U/L]) / ((Plt [10^9/L]) x (ALT [U/L]) (1/2))}
\]

The AUROC of the index was 0.765 for differentiation between Ishak stage 0-3 and 4-6. At a cut off of < 1.45 in the validation set, the negative predictive value to exclude advanced fibrosis (stage 4-6) was 90% with a sensitivity of 70%. A cut off of
>3.25 had a positive predictive value of 65% and a specificity of 97%. Using these cutoffs, 87% of the 198 patients with FIB-4 values outside 1.45-3.25 would be correctly classified, and liver biopsy could be avoided in 71% of the validation group.

5.25 **13C- Caffeine Breath Test**

According to Park et al.\(^{142}\), the properties of caffeine render it an ideal substrate for a quantitative test of liver function. It has high oral bioavailability, and undergoes almost exclusive hepatic metabolism, principally via demethylation by cytochrome P450 1A2 (CYP1A2) to CO\(_2\).\(^{143}\) It has a low extraction ratio and low plasma binding,\(^{144}\) and at the test doses used may be considered innocuous. These characteristics render caffeine an ideal substrate for a breath test of hepatic function.\(^{142}\)

The authors studied that whether the caffeine breath test (CBT) using orally administered \(^{13}\)C-caffeine correlates reliably with plasma caffeine clearance and reflects varying degrees of liver dysfunction. The CBT was performed in 25 healthy controls; 20 subjects with non-cirrhotic, chronic hepatitis B or C; and 20 subjects with cirrhosis.

Cirrhotic patients were characterized by significantly reduced CBT values (1.15 ± 0.75 Δ o/oo mg\(^{-1}\)) compared with controls (2.23 ± 0.76; \(p = 0.01\)), and hepatic patients (1.83 ± 1.05; \(p = 0.04\)). There was a significant inverse relationship between the CBT and Child-Pugh score (\(r = -0.74\), \(p = 0.002\)). The intraclass correlation between repeated CBT’s in 20 subjects with normal and cirrhotic livers was 0.89. Multivariate analysis revealed that only smoking (\(p< 0.001\)) and disease state (\(p = 0.001\)) were significant predictors of CBT.

The authors concluded that the results from their study support the application of CBT as a test of quantitative liver assessment. 1) The elimination of caffeine is impaired in cirrhosis, characterized by a reduction in plasma clearance and a prolongation in caffeine half-life; 2) the oral \(^{13}\)C-CBT using a single 1-hour measure correlates very closely with plasma caffeine clearance; 3) cirrhotic subjects show significantly reduced CBT results compared with control and hepatitis subjects; 4) the CBT values are increased in smokers but still distinguish smokers of varying hepatic functional impairment; 5) the CBT is administered easily and safely in subjects with liver disease; 6) the CBT appears to be a reproducible assay\(^{142}\).
The CBT exhibited significant correlations with serum albumin and platelet count and correlated modestly with INR, bilirubin, γ-glutamyl transferase, and AST/ALT ratio, markers traditionally associated with hepatic dysfunction. The CBT was associated inversely with the Child-Pugh score and differentiated the grades of cirrhosis. In addition, it was able to predict the cirrhotic state in two patients with biopsy proven cirrhosis who had completely normal physical and laboratory findings but significantly reduced CBT values. It thus may be viewed as a complementary test in the overall assessment of liver status, to be taken in context with laboratory, radiologic, and histologic data where available.142

### 5.26 Fibrosis Markers to Assess Effect of Treatment

According to Afdhal N19, an attractive use of fibrosis markers would be to assess the effects of treatment, and if they can be validated, they could be valuable tools in the search of new antifibrotic treatments. However, none of the currently available markers have yet been validated for use in this way, but several studies have shown that levels of these markers are altered by treatment and they have prognostic value.

Poynard and colleagues reported on two studies describing the relationship between response to interferon therapy and the results of Fibrotest performed before and after therapy.78,82 In the larger of these studies (n = 352)82, the authors studied the effect of interferon plus ribavirin on both the Fibrotest and Actitest scores. Actitest is a modification of Fibrotest that incorporates ALT and reflects both liver fibrosis and necroinflammatory activity. All patients had two interpretable liver biopsies and stored serum sample before and after treatment. Of the patients, 208 patients received peginterferon alfa-2b, 1.5 mcg per kg and ribavirin. The remaining 144 patients received interferon alfa-2b, 3 MU and ribavirin. All patients received treatment for 48 weeks.82

At baseline, Fibrotest performed fairly well for the diagnosis of significant hepatic fibrosis, defined as METAVIR score of F2-4. The area under the receiver operating curve was 0.733 ± 0.03, but was lower than that which had been reported in the original study by this group (0.827)54. It increased to 0.766 ± 0.03 at the end of follow up. For bridging
fibrosis and/or moderate necroinflammatory activity, the area under the ROC was $0.76 \pm 0.03$ at baseline and $0.82 \pm 0.02$ at the end of follow up. The index had 90% sensitivity and 88% for the diagnosis of bridging fibrosis and moderate necroinflammatory activity.

Actitest showed slightly greater diagnostic value for the identification of individuals with more advanced fibrosis and with greater histological activity as determined by Knodell score, and may thus be of value in guiding treatment decisions. Of interest, the performance of Actitest improved with longer liver biopsies, those greater than 15 mm or which contained six or more portal tracts, suggesting that these assays truly reflect the more global liver histology.

On follow up, patients who had a sustained virological response to interferon had a substantial reduction in Fibrotest and Actitest scores, as compared to those who were either primary nonresponders or relapsed following therapy. There was a significant decrease of Fibrotest among the 184 sustained virological responders, from $(0.39 \pm 0.02$ to $0.28 \pm 0.02$) at 72 weeks; in comparison with 126 nonresponders from $(0.59 \pm 0.03$ to $0.55 \pm 0.02$) at 72 weeks, $p<0.001$; and with 42 relapsers, from $(0.49 \pm 0.04$ to $0.45 \pm 0.02$) at 72 weeks, $p<0.001$.

There was also a significant decrease of Actitest among the 184 sustained virological responders, from $(0.55 \pm 0.02$ to $0.08 \pm 0.020$) at 72 weeks, in comparison with 126 nonresponders from $0.58 \pm 0.02$ to $0.50 \pm 0.03$ at 72 weeks, and in comparison with 42 relapsers, from $(0.58 \pm 0.03$ to $0.43 \pm 0.03$) at 72 weeks.

Furthermore, there was a significant concordance between Fibrotest and fibrosis stage variations. At baseline 32 patients had cirrhosis. Fibrotest scores fell substantially in 17 patients with cirrhosis at baseline and a post treatment reduction of 1 or more stages; from 0.68 to 0.44 for 3 stages improvement, from 0.60 to 0.47 for 2 stages improvement and from 0.61 to 0.56 for 1 stage improvement. For 15 patients, the fibrosis stage remained F4 and Fibrotest did not significantly change $(0.67 \pm 0.05$ vs $0.64 \pm 0.05$).

These data support the concept that these assays may be useful not only in the initial staging of the liver disease, but may also be of value in following the histological response to therapy.

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According to Afdhal N\textsuperscript{19}, many other studies have evaluated the effect of interferon therapy on levels of fibrosis markers and have compared them to histological findings\textsuperscript{93,105,114,145-153}. Most of these studies have shown that serum levels of several of these markers including HA, PIIINP, YKL-40, and TIMP-1 fall in patients who achieve a sustained virological and biochemical response. In these patients, levels continue to fall following treatment and often return to normal levels. In patients who relapse following treatment, the levels frequently fall during therapy but most often return to pretreatment levels with virological and biochemical relapse\textsuperscript{145}. Where follow up biopsies have been performed, levels of HA and PIIINP have often been found to correlate better with improvement in fibrosis stage, than with biochemical or histological markers of inflammation\textsuperscript{94,105,151,152}. Furthermore, levels of these markers often become normal despite evidence of fibrosis on liver biopsy, suggesting that these markers do reflect the underlying fibrogenic activity within the liver.

According to Afdhal N\textsuperscript{19}, treatment with interferon has been associated with a fall in serum markers of fibrosis, independent of a biochemical or virological response\textsuperscript{145,148,149,153}, while other studies have not confirmed these findings\textsuperscript{93}. And this reduction in the levels of these markers tends to be less than which is observed in patients who achieve a biochemical and virological response. This has been used as evidence that interferon has a beneficial direct antifibrotic effect, presumably through direct inhibition of TGF-β expression\textsuperscript{154,155}.

Serum TGF-β levels and tissue expression has been shown to decline in response to successful therapy of hepatitis C and following treatment of autoimmune hepatitis\textsuperscript{156,157,158}. Based on the available data it is not possible to discern whether interferon has a clinically important antifibrotic effect and hence ongoing long-term low dose interferon studies and studies of interferon –γ and interferon with more potent antifibrotic activity and less antiviral effect are awaited with interest\textsuperscript{19}. 


5.27 Fibrosis Markers to Predict Disease Progression

According to Afdhal N \(^1^9\), if noninvasive markers of fibrosis truly reflect fibrogenic activity and the rate of underlying disease progression then they should have prognostic value, both for predicting progression of fibrosis, and clinical outcomes. In this regard, the available data are more limited. However, several studies looked at the prognostic value of these fibrosis markers in patients with more advanced liver disease \(^1^0^1,1^0^2,1^5^9\).

Guechot evaluated the predictive value of HA in a cohort of 91 patients with hepatitis C associated cirrhosis followed for a median of 38 months \(^1^5^9\). During this time, severe complications including death, liver transplantation, and severe complications of cirrhosis occurred in 24 patients. Of all the laboratory tests evaluated, HA was found to have the greatest predictive value and was equivalent to Child-Pugh score. Similarly, in a cohort of 97 patients with primary biliary cirrhosis, Poupon demonstrated that by multivariate analysis HA, PIIINP, bilirubin, and prothrombin time were independently predictive of disease progression \(^1^0^1\). Interestingly, in the subgroup of 49 patients who were treated with ursodeoxycholic acid therapy, only HA was predictive of a poor outcome.

Nojgaard reported on the ability of PIIINP and YKL-40 to predict survival in a cohort of 370 patients with alcoholic liver disease \(^1^1^5\). In this study, elevated levels of PIIINP and YKL-40 were predictive of shorter survival and carried an increased relative risk of dying of 3.32 (95% CI 1.05-10.5), and 4.24 (95% CI 2.18-8.26), respectively. As prognostic assays, it is not possible to discern whether these markers simply reflect more advanced liver disease or whether they identify individuals whose fibrotic disease is progressing more rapidly and hence have a worse prognosis \(^1^9,1^1^5\).

According to Afdhal N \(^1^9\), serum TGF-β levels have also been used to assess disease progression \(^1^6^0,1^6^1\). In a 12 month study of 39 patients with hepatitis C infection, who underwent paired liver biopsies, Kanzler showed that those patients who had progressive liver fibrosis had higher levels of serum and tissue expression of TGF-β than
those who did not progress\textsuperscript{160}. No such correlation could be found for PIIINP, serum transaminases, or viral load.

Neuman and colleagues\textsuperscript{161}, using a slightly different study design, obtained similar results. They assessed two patient cohorts, a group of 56 patients with minimal or no fibrosis and no inflammation and a second group of 103 patients with no fibrosis but mild histological disease activity. When followed over 3 years, those patients who showed progressive hepatic fibrosis had a parallel increase in TGF-β levels. Hence, TGF-β levels were found to identify the subgroup of patients with mild liver disease that subsequently progressed\textsuperscript{19,161}.

Similarly, prognostic value of the Fibrotest was compared with biopsy staging for predicting cirrhosis decompensation and survival in patients with chronic HCV infection. The investigators concluded that the Fibrotest measurement of HCV biomarkers has a 5-year prognostic value similar to that of liver biopsy\textsuperscript{26}.

According to the authors, Fibrotest was a better predictor than biopsy staging for HCV complications, with the area under the ROC, 0.96 vs 0.91, respectively; it was also a better predictor for HCV related deaths, AUROC 0.96 vs 0.87 respectively. The prognostic value of Fibrotest was also significant in multivariate analyses after taking into account histology, treatment, alcohol consumption, and HIV coinfection\textsuperscript{26}.

\section*{5.28 Gene Markers and Liver Fibrosis}

The hepatic fibrogenesis is a complex process requiring an intricate balance between extracellular matrix (ECM) deposition and removal. Indeed, the ECM metabolism is a very dynamic process, influenced by factors/cytokines/molecules that contribute to its deposition and by others that mediate its degradation\textsuperscript{24}.

The complexity of the fibrogenetic process and the high number of factors/cytokines/molecules involved imply that several genetic polymorphisms could influence progression of liver fibrosis. The genetic polymorphisms linked to hepatic fibrogenesis have been investigated mainly in chronic hepatitis C and in alcoholic fatty
liver disease. There are many studies that report gene polymorphisms to either favor or reduce fibrogenesis in patients with different forms of chronic liver disease. 24

While these studies clearly indicate that many genetic factors have a definitive influence on the risk of developing a more or less active and progressive fibrogenesis, very few of them have found an application as a diagnostic/prognostic marker in clinical practice, due to their complexity, difficulty to test and variable behavior in different patient populations. 24

One such study, consists of a set of seven marker genes, the cirrhosis risk score (CRS), was found to be a better predictor of high risk versus low risk for cirrhosis in Caucasian patients than clinical factors. 162 The clinical factors such as age, gender, alcohol use, and age at infection, obesity and hepatic steatosis, 163-165 influence the progression to cirrhosis, but cannot accurately predict the risk of developing cirrhosis in patients with chronic hepatitis C. 16

The authors hypothesized that host genetic factors, such as single nucleotide polymorphisms (SNP’s) could play a primary role in determining fibrosis risk. The preliminary results from their previous study suggested that replicated SNP’s identified by large association studies had several advantages over clinical risk factors, such as higher odds ratios, consistent percentages of risk population across different study cohorts, and objective genotyping calls that are available for all patients. 166 The key question remained as to how these markers could be utilized in clinical settings.

The aim of the study was to develop and validate a predictive signature (of genes) alone or in combination with standard clinical factors to assess the risk of cirrhosis in Caucasian patients with chronic hepatitis C virus infection. 162

In the training set (n = 420), all patients had well characterized liver histology and clinical factors. DNA was extracted from the whole blood for genotyping. The authors validated all significant markers from a genome scan in the training cohort, and selected 361 markers for the signature building. Subsequently, a signature consisting of 7 markers most predictive for cirrhosis risk in Caucasian patients was developed. The Cirrhosis Risk Score (CRS) was calculated to estimate the risk of developing cirrhosis for each patient. The CRS performance was then tested in an independently enrolled validation cohort of 154 Caucasian patients.
The area under the receiver operating characteristic (ROC) curve was 0.75 in the training cohort. In the validation cohort, the ROC was only 0.53 for clinical factors, increased to 0.73 for CRS, and 0.76 when CRS and clinical factors were combined. A low CRS cutoff of < 0.50 to identify low risk patients would misclassify only 10.3% of high risk patients, while a high cut off of > 0.70 to identify high risk patients would misclassify 22.3% of low risk patients. Thus more importantly fewer of the high risk patients would be misclassified. In conclusion, CRS is a better predictor than clinical factors in differentiating high risk versus low risk for cirrhosis in Caucasian patients \(^{162}\).

According to the authors, for the first time, one can estimate the risk of cirrhosis rather than using findings of liver biopsy to project the future course of disease. Liver biopsy represents only one time point in the long natural history of hepatitis C, whereas the genetic markers are intrinsic and life long. Potentially the CRS could be used to stratify patients’ cirrhosis risk prior to liver biopsy \(^{162}\).

Unlikely rare Mendelian disorders, and similar to other complex human diseases, liver cirrhosis is caused by the interactions among multiple genetic and environmental factors. Yang et al, estimated that for genes with very common genotype frequencies (>30%) and moderate OR’s (1.2-1.5), 10-15 markers are needed to achieve appreciable population attributable fraction (PAF) for disease occurrence \(^{167}\). Consistent with this finding, the CRS signature is comprised of seven markers with high frequencies (18.5%-87.3%) and significant OR’s (1.86-3.23). In contrast, only two clinical factors, sex and age, had significant associations with cirrhosis risk. Moreover, the CRS measurements are objective \(^{162}\).

In the CRS, each of the 7 most predictive markers only provide moderate predictability; whereas the combination of these 7 was more robust and predictive. This finding is consistent with multiple biological pathways known to be involved in hepatic fibrogenesis \(^{168}\).

Of the 7 genes, antizyme-Inhibitor-1 (AZIN1) and Toll-like receptor 4 (TLR4) have an identified role in hepatic fibrosis. AZIN1 binds to ornithine decarboxylase (ODC) antizyme and stabilizes ODC, thus inhibiting antizyme-mediated ODC degradation \(^{169}\). Regarding TLR4, it is expressed in all hepatic cell types in response to its ligand lipopolysaccharide (LPS) \(^{170}\). In patients with chronic hepatitis C, NS5A induces
the elevated expression of TLR4 in B cells 3- to 7- fold. Moreover, LPS-TLR4 pathway plays an important role in the hepatic fibrogenesis. The authors were in the process of determining the functional mechanisms of the other 5 genes in fibrogenesis, and the applicability of the CRS in other liver diseases.

Nevertheless, improved understanding of the genetic influence on liver fibrogenesis is of paramount importance as it may lead in the near future to identification of patients for high risk for fibrosis/cirrhosis, diagnosis of liver fibrosis, and new therapeutic targets and strategies for development of effective antifibrotic treatments.

5.29 Radiology in the Noninvasive Assessment of Liver Fibrosis

According to Afdhal N, radiological evaluation of liver to assess fibrosis has been limited to the identification of individuals with cirrhosis and its complications. The advent of cross sectional imaging with CT, MRI, and ultrasound enables detailed images of the liver and surrounding structures to be made. Resolution of the hepatic parenchyma with any of the available modalities, however, is insufficient to determine any of the earlier stages of fibrosis before the establishment of cirrhosis and portal hypertension.

Established cirrhosis with portal hypertension can be determined with a high specificity by identifying splenomegaly, an enlarged caudate lobe, or the presence of large varices. Studies using CT, ultrasound, and MRI, have identified reduction in the size of the right lobe of the liver with relative enlargement of the left and caudate lobes as reliable markers of cirrhosis.

Harbin’s original description of this technique measured the ratio of transverse width of the caudate lobe, to the transverse width of the right lobe of the liver. Using this technique, cirrhosis could be correctly diagnosed with sensitivity 84%, a specificity of 100%, and diagnostic accuracy of 94%. These studies report a high specificity but limited sensitivity because these morphological changes are only present in more advanced disease.

Further studies using up to 11 sonographic and Doppler parameters, including: measurements of liver morphology, assessment of portal venous blood flow, spleen size,
and liver surface nodularity; reported accuracy of cirrhosis detection between 82% and 88\% \textsuperscript{175,176}, however anatomical limitations and interobserver variability remained limiting factors \textsuperscript{39}. In addition, it should also be noted that a large number of patients had decompensated liver disease, and the diagnosis of cirrhosis could have been made using simple clinical criteria, including palpation of a nodular liver, or the presence of a hard and enlarged left lobe \textsuperscript{175}.

When Oberti compared the ability of ultrasound to diagnose cirrhosis to clinical examination and biochemical studies, he found ultrasonography to be of less diagnostic value \textsuperscript{70}.

It should be noted that ultrasound also identified cirrhosis in a substantial number of patients in which a definite diagnosis of cirrhosis was not made on liver biopsy, in the study cited above \textsuperscript{175}. It can therefore, be argued that ultrasound examination has substantial complementary value, i.e., a properly performed examination can identify patients with cirrhosis where the biopsy findings are equivocal, or at variance with clinical impression \textsuperscript{19}. Furthermore, progression in the ultrasonographic changes, including an enlarging spleen has been associated with an increased risk of complications\textsuperscript{177}.

5.30 Measurement of Hepatic Stiffness (Elastography)

Accepting the limitations of standard radiological techniques to accurately determine fibrosis, transient elastography is an emerging technology that is more sensitive for staging hepatic fibrosis. This technique rapidly and noninvasively measures mean hepatic tissue stiffness \textsuperscript{178,179}.

Hepatic stiffness is related to the degree of fibrosis; palpation of a firm liver edge has been used for centuries as a marker of hepatic injury. Using a probe (Fibroscan, Echosens, Paris) that includes an ultrasonic transducer, a vibration of low frequency (50 MHz) and amplitude is transmitted into the liver. The vibration wave induces an elastic shear wave that propagates through the organ. The velocity of this wave as it passes through the liver correlates directly with tissue stiffness and by simultaneously using a
pulse-echo ultrasound by means of the same probe, the velocity of the wave is measured. The harder or stiffer the tissue, the faster the shear wave propagates. Results are expressed in kilopascals (kPa). Fibroscan measures liver stiffness of a volume that is approximately a cylinder of 1 cm diameter, and 2 cm long, 100 times greater in size than a standard liver biopsy. Thus it is more representative of the entire hepatic parenchyma.

According to Afdhal N 19, initial studies of hepatic elastography in 19 hepatectomy specimens correlated with histologic analysis 180. In vivo study of a cohort of 15 patients with hepatitis C virus (HCV) showed excellent intra- and inter-operator reproducibility.

Furthermore, in 91 patients who had HCV reported by the same authors, the receiver operating characteristic (ROC) curve, which estimates the diagnostic performance of elastography, were 0.90, 0.88, 0.91, and 0.99, respectively, for the hepatic fibrosis grade superior and equal to F1, F2, F3, and F4, respectively. Of the studied patients with a score of less than 5.1 kPa, 93% were METAVIR F0 or F1, whereas for those with a score of 7.6 kPa or higher, 94% were F2 or more 178.

In a large multicenter study 41, the authors enrolled 327 patients. The aim of the study was to investigate the use of liver stiffness measurement in the evaluation of liver fibrosis in patients with chronic hepatitis C. Patients underwent both liver biopsy and liver stiffness measurement. METAVIR liver fibrosis stages were assessed on biopsy specimens.

The areas under the receiver operating characteristic curve (ROC); for $F \geq 2$ was 0.79 (95% CI, 0.73-0.84), for $F \geq 3$ was 0.91 (0.87-0.96), and for $F \geq 4$ was 0.97 (0.93-1.0). For larger biopsy specimens (longer than the median value in each category), these values were, 0.81, 0.95, and 0.99 respectively. Optimal cut of values of 8.7 and 14.5 kPa showed $F \geq 2$, and $F = 4$, respectively 41.

The authors concluded that they found significant positive correlation between liver stiffness measurement and fibrosis stages in patients with chronic hepatitis C. Although there was overlap in scores for those with early stages of fibrosis; yet there was significant areas under the ROC curves for $F \geq 3$, and $F = 4$ fibrosis, and with high total sensitivity, specificity, and high likelihood ratios suggest that liver elastometry is a
reliable method for the diagnosis of extensive fibrosis ($F \geq 3$), and cirrhosis ($F = 4$). In the study 30% of patients were $F \geq 3$, or $F = 4$. However, in the community practice, where the proportion of patients with $F4$ may be lower than in referral centers, liver stiffness measurement accuracy in predicting patients with $F2$ or more METAVIR fibrosis stage might be lower $^{41}$.

The study also confirmed that in chronic hepatitis C patients, the correlation between liver stiffness and fibrosis stage is not affected by steatosis or activity grade. Indeed, activity was not expected to modify the liver stiffness, whereas steatosis could have been expected to soften the liver because it consists of fat deposits in the liver parenchyma. But in the study the multivariate analysis showed that the potential effect of steatosis on liver stiffness was hidden by the strong effect of fibrosis. These findings support a study on elastic modulus measurements of ex vivo human liver samples that reported a correlation between liver stiffness and fibrosis but did not show any obvious correlation between steatosis and elastic modulus $^{180}$.

Results show that the diagnostic performances of liver stiffness measurement were better in the larger specimens than in the smaller specimens. This suggests that the real diagnostic performance of liver elastometry may be underestimated because of the sampling error of the biopsy $^{41}$.

According to the authors $^{41}$, the diagnostic performance of liver elastometry appears to be equivalent to that of the best biochemical scores for patients with significant fibrosis ($F \geq 2$), and is better than these tests for the diagnosis of extensive fibrosis ($F \geq 3$) and cirrhosis ($F = 4$), (there are studies which report better performance of serum markers in earlier stages). The main advantage of liver elastometry over the fibrosis markers is that it measures a quantitative physical parameter directly on the liver and there is no interference from extrahepatic disorders. It, therefore, is complementary to the fibrosis markers to better assess liver fibrosis without resorting to liver biopsy $^{41}$.

There are certain limitations to the procedure. Elastometry cannot be applied to patients with ascites, even if clinically undetected. Ascites is a physical limitation to the technique because elastic waves do not propagate through the liquids. In addition, elastometry is unsuccessful in patients with narrow intercostals spaces and in patients with morbid obesity. In obese patients the fatty thoracic belt attenuates both elastic waves
and ultrasound, rendering liver stiffness measurement more difficult or even impossible. Probes with smaller size and elongated shaped transducer tips are currently available for these patients.\(^{41}\)

In another study, a total of 711 patients with chronic liver disease were evaluated. In cirrhotic patients, liver stiffness measurements range from 12.5 to 75.5 kPa. However, the clinical relevance of these values is unknown.

The aim of the prospective study was to evaluate the accuracy of liver stiffness measurement for the detection of cirrhosis in patients with chronic liver disease\(^ {181}\). Etiologies of chronic liver diseases were hepatitis C virus or hepatitis B virus infection, alcohol, non-alcoholic steatohepatitis, other, or a combination of the above etiologies. Liver fibrosis was evaluated according to the METAVIR score.

Areas under the receiver operating characteristic curve were 0.80 for patients with significant fibrosis (F>2), 0.90 for patients with severe fibrosis (F3), and 0.96 for patients with cirrhosis. Using a cut off value of 17.6 kPa, patients with cirrhosis were detected with a positive predictive value and a negative predictive value (NPV) of 90%. Liver stiffness was significantly correlated with complications of liver disease. With an NPV >90%, the cut off values for the presence of esophageal varices stage 2/3, cirrhosis Child-Pugh B or C, past history of ascites, hepatocellular carcinoma, and esophageal bleeding were 27.5, 37.5, 49.1, 53.7, and 62.7 kPa, respectively\(^ {181}\).

The authors concluding that transient elastography is a promising non-invasive method for detection of cirrhosis in patients with chronic liver disease. Its use for the follow up and management of these patients could be of great interest and should be evaluated further.

The limitation is that it requires a costly device to measure liver stiffness\(^ {24}\). Similarly, some of the direct markers of noninvasive evaluation of fibrosis are only available in highly specialized research laboratories and are thus not routinely available.

Thus in conclusion, this simple noninvasive technique has proved beneficial in detecting patients with advanced fibrosis or cirrhosis and more generally in assessing fibrosis in patients with chronic hepatitis C.

Studies underway will evaluate the applicability of hepatic elastography across a range of liver diseases. Further research also will determine its use for assessing hepatic
fibrosis over time and its responsiveness to detecting changes in fibrosis associated with specific therapies, in particular the response to antiviral therapies for chronic viral hepatitis. For now, the preliminary results and its ease of use predict a promising future in the reliable staging of fibrosis 39.
5.31 Platelets in Chronic Liver Disease – The role of Spleen

Thrombocytopenia is a commonly encountered hematological condition in chronic liver disease and cirrhosis. It is a risk factor for gastrointestinal bleeding and other life threatening hemorrhagic events.

Historically it has been attributed to the sequestration and destruction of platelets in the enlarged spleen with an impaired ability of bone marrow to compensate by increasing platelet production. Hypersplenism occurs in a varying percentage of patients with advanced liver disease and is a common complication of portal hypertension. Radiolabeled platelet studies have shown that human spleen contains a sizeable fraction (about one third) of the total body platelet mass in the form of exchangeable pool. The spleen can increase its pool by 30-90% when pathologically enlarged. This redistribution of cells from the peripheral circulation to the spleen appears sufficient to produce thrombocytopenia despite the normal platelet life span, normal total body mass and unimpaired platelet production, as might be expected form the number of megakaryocytes in the marrow.

However attempts to correct the low platelet levels by splenectomy and portal decompression procedures have failed to consistently improve the platelet count in the long term.

5.32 Platelet Autoantibodies and Thrombocytopenia

It has been postulated that auto-antibody mediated platelet destruction, as seen in patients with idiopathic thrombocytopenia purpura (ITP) may also contribute to cirrhotic thrombocytopenia. In this situation, the thrombocytopenia is principally mediated by enhanced platelet clearance in the periphery, resulting in accelerated platelet turnover. Autoimmune mechanism mediated by platelet associated Ig may play an important role in thrombocytopenia associated with viral hepatitis.
In another study, antiplatelet autoantibodies were determined in blood serum with the use of ELISA method in 15 patients with cirrhosis and thrombocytopenia (mean platelet count 67.9 +/- 24.9 x 10^3/µl). Three patients (20%) presented with anti-GPIIb/IIIa antibodies and 2 patients with anti-GPIa/IIa. These patients had liver failure (stage C according to Child-Pugh classification) and splenomegaly. Platelet morphological parameters were also evaluated. The significant decrease of plateletcrit as well as the decrease of mean platelet volume (MPV) was observed in liver cirrhosis with thrombocytopenia. The increase of megathrombocyte population (MPV > 20fl) up to 5.5% of all platelets was also observed. Megathrombocytes in healthy individuals were 2.25% of platelet population. Examinations confirmed that autoimmunological factors play an important role in the development of thrombocytopenia in liver cirrhosis.

The authors postulating that immunological disorders in patients with liver cirrhosis, loss of tolerance to own antigens, and the change of platelet antigenicity enable antiplatelet antibody formation under the influence of continuous activation.

Another study has shown that serum thrombopoietin levels may not be directly associated with thrombocytopenia in patients with chronic hepatitis and liver cirrhosis. In contrast spleen volume and platelet associated immunoglobulin (PAIgG) are associated with thrombocytopenia in such patients, suggesting that hypersplenism and immune mediated processes are predominant thrombocytopenic mechanisms.

5.33 Thrombopoietin and Chronic Liver disease

Impaired platelet production due to thrombopoietin (TPO) deficiency has also been proposed as another cause of thrombocytopenia in cirrhosis patients. Thrombopoietin, a principal regulator of megakaryopoiesis is predominantly produced by the liver. Its levels are insufficient in advanced liver failure. This is further supported by the observation that reduced circulating level of TPO in cirrhosis patients is restored in conjunction with an increase in platelet count after orthotopic liver transplantation.
Adinolfi et al., has shown that in patients without splenomegaly, the thrombocytopenia was associated with the stage of fibrosis; platelet counts were the highest in patients with fibrosis stage 0-2 (Knodell HAI), lower in those with stage 3 (p< 0.008) and lowest in those with stage 4 (p< 0.05). These findings were independent of demographic, biochemical, hepatic necroinflammatory activity, portal hypertension and splenomegaly. Patients with normal platelet counts showed higher thrombopoietin levels than those with low platelet counts (p< 0.0001). An inverse correlation between thrombopoietin levels and fibrosis grade was observed (r= -0.50; p, 0.0001). The authors postulated that advanced hepatic fibrosis causing an altered production of thrombopoietin and portal hypertension, plays a central role in the pathogenesis of thrombocytopenia in chronic viral hepatitis 87.

Evaluating the association between the degree of fibrosis and the platelet count, another study included seven hundred eighty-four patients (265 chronic viral hepatitis C and 519 chronic viral hepatitis B). In an effort to avoid the effects of hypersplenism, patients with splenomegaly and/or bi- or pancytopenia were excluded. In multivariate analysis, the peripheral platelet count had a negative correlation with the fibrosis score and age, but not with necroinflammatory activity, in both groups. The authors concluding that, a decrease in peripheral platelet count may be a sign of an increase in the degree of fibrosis during the course of chronic viral hepatitis B and C and factors other than hypersplenism may play a role in this decrease in the peripheral platelet count 193.

However, several other studies have found that the circulating TPO level is maintained or even increased in patients with cirrhosis 86,194 including the study by Kajihara et al182.

In their study when compared with healthy controls, cirrhosis patients presented with, (i) normal or slightly increased plasma TPO, (ii) accelerated platelet turnover based on elevated %RP (reticulated platelets) and GCI (glycocalcin index), and (iii) reduced platelet production based on decreased absolute RP count and plasma GC 182.

Reticulated platelets (RP) are young platelets that contain higher levels of nucleic acid components than mature platelets. The absolute RP count is a reliable indicator of the thrombopoiesis rate, analogous to the reticulocyte count to evaluate erythropoiesis. Glycocalcin is a proteolytic fragment of the α-chain of glycoprotein (GP) Ib, which is
cleaved from the surface of megakaryocytes and platelets. The plasma glycocalcin concentration is decreased in patients with aplastic anemia and greatly increased in patients with essential thrombocythemia, indicating that it is a marker of platelet production\textsuperscript{195}.

In contrast, the proportion of reticulated platelets in total platelets ($\%$RP) and the plasma glycocalcin level normalized to the individual platelet count (GC Index; GCI) have been shown to reflect platelet turnover\textsuperscript{196}.

These markers in ITP and cirrhosis patients were comparable, but significantly different from those in aplastic anemia patients. The bone marrow megakaryocyte density in cirrhosis and ITP patients was similar, and significantly higher than in aplastic anemia patients\textsuperscript{182}.

The authors concluded that cirrhotic thrombocytopenia is a multifactorial condition involving accelerated platelet turnover and moderately impaired thrombopoiesis. Thrombopoietin deficiency is unlikely to be the primary contributor to cirrhotic thrombocytopenia\textsuperscript{182}.

It has also been shown in one of the studies that in acute liver failure (ALF) the inverse relationship between platelet count and TPO levels was not observed. Despite severe hepatic dysfunction, serum TPO levels were initially normal and increased during hospitalization in acetaminophen-induced ALF, but did not prevent the development of thrombocytopenia\textsuperscript{197}.

TPO is synthesized primarily in the liver as a single 353-amino acid precursor protein. Following removal of the 21 amino acid signal peptide, the remaining 332 amino acids undergo glycosylation to produce a 60-70 kDa protein. Thrombopoietin is produced at a constant rate by the liver and enters the circulation where most of it is removed by avid thrombopoietin (c-mpl) receptors on normal platelets. The residual amount of thrombopoietin (50-150 pg/ml) provides basal stimulation of megakaryocytes and a basal rate of platelet production\textsuperscript{198}.

It is the circulating platelet mass, not the platelet count, which is regulated by the body. A practical demonstration of this principle is the effect of changes in the size of the spleen, (which normally sequesters one third of the platelet mass), on the platelet count. With increasing splenomegaly, thrombocytopenia becomes progressively more severe,
but the total body mass of platelets (circulating + splenic pools) remains constant. How the body maintains this constant circulating platelet mass has been the subject of considerable investigation\textsuperscript{198}.

Thrombopoietin is released into the circulation at a constant rate. In the absence of platelets, there is little clearance of thrombopoietin by platelets, levels rise, bone marrow megakaryocytes are stimulated and platelet production increases. In contrast, in the presence of platelets, thrombopoietin clearance increases, levels are low, megakaryocytes are not stimulated and basal platelet production ensues. Unlike the mechanism for red blood cells, there is no "sensor" of the platelet mass; instead, as occurs in the regulation of neutrophils and monocytes where the regulated cells bind and clear their regulatory cytokine, the circulating platelet mass directly determines the circulating level of thrombopoietin\textsuperscript{198,199}.

Since it is the total number of circulating platelet c-mpl receptors that determines the clearance of thrombopoietin, the constancy of normal circulating platelet mass, and not the platelet count can now be explained. Which is the body defends the total mass of platelets, and not the platelet count. The platelet count decreases proportionally to the increase in the size of spleen, but the total mass of platelets remains normal and unchanged. This could be one explanation for low platelets and not having increased thrombopoietin levels.

5.34 Platelet Parameters and Chronic Liver Disease

The quantitation of platelets in peripheral blood is a well-recognized tool. Recently, new indices related to platelet counts have been provided by hematologic analyzers. Concerning the platelet parameters, the important parameters are mean platelet volume (MPV), platelet distribution width (PDW)\textsuperscript{200}. The evaluation of these two parameters does constitute part of the routine evaluation of complete blood count (CBC) in modern automated analyzers, but clinical inferences are usually not drawn. However, there are interesting observations.
One observer, found a significant correlation between PDW and RDW. And the anisocytosis of red blood cells and platelets might co-occur. However, these data are basic observations; further in-depth evaluation of the platelet parameters is recommended.

The mean platelet volume is the geometric mean of the transformed lognormal platelet volume data in impedance technology systems. In some, optical systems, (e.g., Bayer), MPV is the mode of the measured platelet volume.

Under normal circumstances, there is an inverse relationship between platelet size and number. Therefore, the total platelet mass, the product of MPV and the platelet count (plateletcrit) is closely regulated. When platelets decrease in number, bone marrow megakaryocytes are stimulated by thrombopoietin and produce larger platelets. Thus platelets with a larger volume are expected to be seen in destructive thrombocytopenia when megakaryocytic stimulation is present. Conversely, platelets with low MPV are expected to be seen in thrombocytopenic states seen in marrow hypoplasia or aplasia.

A very important exception occurs in splenic sequestration, in which a low MPV is seen, because spleen sequesters large platelets.

A decrease of mean platelet volume (MPV) was observed in liver cirrhosis with thrombocytopenia. This fact has been known from before, for instance in a study from 1984, MPV in patients with liver disease (9.25 ± 1.14 fl) was significantly lower than that of controls (10.52 ± 0.74 fl, p< 0.001). In control subjects, the MPV and platelet counts were inversely correlated.

It has also been shown that, platelet distribution width is a better indicator of altered platelet homeostasis than MPV in liver cirrhosis. In the study, platelet count, mean platelet volume, giant platelet percentage, expressed as megathrombocytic index (MTI), and platelet distribution width (PDW) were evaluated in 32 controls, and in 27 patients with cirrhosis and thrombocytopenia. MTI and PDW were linearly and inversely correlated to platelet count both in controls and patients. MTI and PDW were markedly increased in cirrhosis as compared to controls, while MPV was not significantly different. The authors concluded that: MTI and PDW were good indicators of thrombopoietic stimulus both in controls and in cirrhosis and are better indicators of altered platelet homeostasis than MPV in cirrhosis.
These platelet parameters are altered in similar direction in immune thrombocytopenia, as in cirrhotic thrombocytopenia. Likewise, it has been postulated that auto-antibody mediated platelet destruction, as seen in patients with idiopathic thrombocytopenia purpura (ITP) may also contribute to cirrhotic thrombocytopenia \(^{186}\), as already discussed.

In a study the authors, investigated the significance of the platelet indices, mean platelet volume (MPV), platelet size deviation width (PDW), and platelet-large cell ratio (P-LCR), in the diagnosis of thrombocytopenia by comparing these levels in 40 patients with hypo-productive thrombocytopenia (aplastic anaemia; AA) and 39 patients with hyper-destructive thrombocytopenia (immune thrombocytopenia; ITP). The sensitivity and specificity of platelet indices to make a diagnosis of ITP were also compared.

All platelet indices were significantly higher in ITP than in AA, and platelet indices showed sufficient sensitivity and specificity. The area under the curve (AUC) of the receiver operating characteristics curve of platelet indices was large enough to enable the diagnosis of ITP. P-LCR and PDW had the largest AUCs, which indicated that these values were very reliable for immune thrombocytopenia. The authors concluded that, these indices provide clinical information about the underlying conditions of thrombocytopenia. More attention should be paid to these indices in the diagnosis of thrombocytopenia \(^{204}\).
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PATIENTS
and
METHODS
PATIENTS

Inclusion criteria

One hundred patients, aged between 20 and 50 years, with chronic liver disease who were tested positive with HCV RNA PCR and/or HBsAg, and had liver biopsy done for evaluation purposes were consecutively enrolled from the medical outpatient department in this cross sectional study. The patients had increased ALT for more than 6 months. Patients whose PCR remained positive after antiviral therapy were also enrolled, provided they had liver biopsy done within the last 06 months. All patients gave informed consent to enroll in the study and the study protocol was approved by the Board of Advanced Studies and Research (BASR) of the Baqai Medical University, which included the Ethics committee.

Exclusion criteria

Patients with following characteristics were excluded from the study: chronic liver disease other than HBV and HCV, decompensated cirrhosis (modified Child-Pugh class C), prior antiviral therapy and PCR negative afterwards. Patients with insufficient liver biopsy specimen (adequate specimen was taken as containing $\geq 8$ portal tracts or if significant pathology was visible otherwise as assessed by two histopathologists), and incomplete data on noninvasive markers were also excluded from the final analysis. No patient had history of alcoholism, HIV or had liver transplantation.

METHODS

Clinical Data

The clinical data recorded were: age, gender, ethnicity, possible mode of transmission, previous antiviral treatment and result, symptoms of decompensated liver disease, body mass index (BMI), hepatomegaly, splenomegaly, and stigmata of chronic liver disease.
Laboratory Investigations

The lab investigations included: total bilirubin, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Gamma glutamyl transferase (GGT), albumin, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, urea, creatinine and blood sugar random. The analyses for these variables were performed on microlab 200 using commercial reagents. High control, normal control and low control sera were run before and during the analysis of each batch. Globulins and Immunoglobulin G levels were measured by protein electrophoresis. Prothrombin time (PT), international normalized ratio (INR), and partial thromboplastin time with kaolin (PTTK) were performed using commercial tissue thromboplastin reagent and results were interpreted visually. Hyaluronic acid and alpha 2 macroglobulin assays were performed on sera stored at -20°C.

Total leucocyte count, haemoglobin concentration, mean corpuscular volume (MCV), platelet count, mean platelet volume (MPV), and platelet distribution width (PDW) were measured on automated haematology analyzer, Sysmex KX-21. Control cells were run at the start of each day. Osmotic fragility test as an indirect marker of erythrocyte rigidity, was performed in varying dilutions of isotonic saline and was interpreted visually. Haemolysis was ascertained by haemoglobin estimation of supernatant fluid. Percentage of giant platelets was assessed by slide examination of peripheral blood smear stained with Leishman’s stain.

Hyaluronic Acid

The hyaluronic acid (HA) assay was performed on the 96 well microplate (Corgenix Inc., CO, USA). It is an enzyme-linked binding protein assay that uses a capture molecule known as hyaluronic acid binding protein (HABP). Properly diluted serum or plasma and HA reference solutions were incubated in HABP-coated microwells, allowing HA present in samples to react with the immobilized binding protein (HABP). After the removal of unbound serum molecules by washing, HABP conjugated with horseradish peroxidase (HRP) solution was added to the microwells to form complexes
with bound HA. Following another washing step, a chromogenic substrate of tetramethylbenzidine and hydrogen peroxide was added to develop a colored reaction. The intensity of the color was measured in optical density (O.D.) units with a spectrophotometer at 450 nm. HA levels in patients and control samples were determined against a reference curve prepared from the reagent blank (0 ng/ml), and the HA reference solutions, both provided with the kit.

**Alpha 2 Macroglobulin**

Alpha 2 macroglobulin assay was performed on the 96 well polystyrene microplate (Assaypro, MO, USA). The assay employed a quantitative sandwich enzyme immunoassay technique which measured alpha 2 macroglobulin in 4 hours. A polyclonal antibody specific for alpha 2 macroglobulin was pre-coated onto a microplate. Alpha 2 macroglobulin in standards and samples was sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for alpha 2 macroglobulin, which was recognized by a streptavidin-peroxidase conjugate. All unbound material was then washed away and a peroxidase enzyme substrate added. The color development stopped and the intensity of the color measured.

**HBsAg**

In this assay, samples were added to microplate wells pre-coated with monoclonal antibodies specific for HBsAg. During the course of the assay, the positive controls, negative controls were also added. The microtiter plate wells were thoroughly washed to remove unbound other components of the sample. A standardized preparation of horseradish peroxidase (HRP) conjugated antibody specific for HBsAg was added to each well to “sandwich” the antibodies immobilized during the first incubation.

Following a wash to remove any unbound HRP conjugate, a TMB (3, 3’, 5, 5’ tetramethylbenzidine) substrate solution was added to each well. The enzyme (HRP) and substrate were allowed to react over a 10-minute incubation period. The enzyme-substrate reaction was terminated by the addition of sulfuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450nm ± 2nm. Only
those wells containing HBsAg and HRP conjugate exhibited a change in color. The intensity of this color change was proportional to the concentration of HBsAg in the sample.

**Antibody to Hepatitis B e Antigen**

The test was based on Microparticle Enzyme Immunoassay (MEIA) technology. It utilized the principle of competitive binding between anti-HBe in the sample and anti-HBe coated on the microparticles and in the Anti-HBe: Alkaline Phosphatase Conjugate for a standardized amount of Neutralizing Reagent [recombinant DNA derived HBeAg (rHBeAg)].

**HBeAg**

The test was also based on Microparticle Enzyme Immunoassay (MEIA) technology, and utilized the principal of direct binding of the HBeAg in the sample to the anti-HBe coated on the microplates, followed by the detection of the bound HBeAg by the anti-HBe: Alkaline Phosphatase Conjugate.

**Antibody to Hepatitis C Virus**

The recombinant HCV antigen was coated on the multiple wells. The serum sample and Anti-human IgG labeled with HRP (conjugated) were added to the coated wells, and a complex of HCVAg-Anti-HCV-Anti-human IgG labeled with HRP was formed. This enzyme reaction produced a color change, and the intensity of the absorbance at 450 nm indicated the presence or absence of Anti-HCV in the sample.

**RT RNA PCR**

Serum separated within 6 hours and frozen at -70°C was used to perform RT RNA PCR with Amplicor Roche reagent kit. The test utilized reverse transcription of target RNA to gene rate complementary DNA (cDNA). Amplification of target c DNA by polymerase chain reaction and nucleic acid hybridization for detection of HCV RNA in serum was performed.
HBV DNA PCR

HBV viral genome was determined by using PCR-restriction fragment length polymorphism of the surface genome of HBV. Minimum of 10 copies of HBV DNA per specimen by testing serial 10-fold dilutions of HBV transcripts with known amounts (10^8 copies/ml) was considered positive. The DNA was extracted from sera by using a Qiagen (Hilden, Germany) viral DNA kit or equivalent. Amplification was performed in 18 µL of Light Cycler DNA Master SYBER Green or equivalent. 1 mix contained 3.5 mM of Mg Cl₂ by using 2 µL of c DNA and appropriate primers. PCR was performed in 45 cycles of 1 s at 95°C (denaturation), 3 s at 55°C (annealing), and 8 s at 72°C (extension).

Histopathological evaluation

Liver biopsy was performed by using the 18 gauge Surecut liver biopsy needle (modified Menghini liver aspiration needle) or as otherwise mentioned, using the subcutaneous intercostal approach. Prior to liver biopsy informed consent was obtained, and blood CP, PT/INR and PTTK was done. Ultrasound abdomen was carried out to mark the biopsy site, and to rule out any anatomical abnormality of the gall bladder, or silent lesion (such as haemangioma) within the liver parenchyma. Similarly chest X-ray was done to rule out Chiladiti Syndrome (the interposition of bowel between the inner intercostal wall and liver parenchyma).

All biopsy material slides were stained with haematoxylin and eosin and reticulin stains. The biopsies were interpreted by qualified histopathologists (B.A. and Y.W.; with 12 and 10 years experience in liver histopathological reporting respectively). The liver biopsy was scored according to the Knodell HAI classification (mainly), and in few circumstances by modified Knodell HAI system (Ishak scoring system). The histopathologists were blinded to the patient’s clinical and laboratory profile. The biopsy material was considered adequate if it contained ≥ 8 portal tracts or if significant pathology was visible otherwise.

Fibrosis was scored according to Knodell HAI: stage 0, no fibrosis; stage 1, fibrous portal expansion; stage 3, bridging fibrosis (portal-portal or portal- central
linkage); and stage 4, cirrhosis. In Ishak scoring system, stage 2 was also recognized corresponding to fibrous expansion of most portal areas with or without short fibrous septa. Minimal fibrosis was defined as stage 0 or 1, significant fibrosis was defined as stages 2, 3, and 4. Advanced fibrosis was considered for stages 3, and 4. The necroinflammatory activity was graded according to the respective scoring system.

Following the recommendations of International Association for the Study of Liver (IASL) panel, the diagnostic line of pathology report also read the pathologist’s visual impression in terms of mild/moderate/severe (extensive) activity and fibrosis.

**Ultrasonographic parameters**

Ultrasound examination of abdomen was done using the Toshiba ECO-CEE machine, evaluating liver size/echotexture/nodularity, presence or absence of any focal lesion, portal vein diameter and splenomegaly.

**Statistical Analysis**

Statistical analysis was done by logistic regression, receiver operating characteristic (ROC) curves, and by descriptive statistics; sensitivities, specificities, positive and negative predictive values, and diagnostic accuracy. The main end point was the identification of clinically significant fibrosis (F2-F3-F4) from insignificant fibrosis (F0-F1). In the secondary analysis, recognition of moderate to severe necroinflammatory activity from no to mild activity was also included. In addition, different platelet parameters were separately studied for their association with advanced fibrosis (F3-F4).

Firstly, the association between different biochemical markers for the presence or absence of clinically significant fibrosis (CSF; F2-F3-F4), was assessed in univariate analysis. Continuous variables were compared with the student t-test, while categorical variables were compared by the Chi-square test. A 2-sided p value of less than 0.05 was considered statistically significant. The quantitative variables were expressed as mean ± SD, or median in certain circumstances, and as interquartile range in box plots. The categorical variables were expressed as percentages.
In addition, the strength of association of individual biochemical markers with CSF was also assessed by the ROC curve analysis.

The biochemical markers with strong association in univariate analysis, and high area under the ROC curve were then subjected to multivariate analysis. The independent discriminative value of markers for the diagnosis of fibrosis was then assessed by logistic regression analysis. The best index for discrimination was the logistic regression function that combined the most discriminatory independent factors. These markers were then combined with age and sex and entered into a forward step wise logistic regression analysis to determine a probability index ranging from 0 to 1. The dependent variable was the presence or absence of CSF.

A central cut off of 0.5 was chosen for the prediction of dependent variable. The scores presented were those of the study population where β coefficients of score probability were provided by running multivariate analysis of composite variables. The biochemical markers with the best discriminatory value were combined in various logistic regression analyses to develop different models to predict CSF. These models were then compared amongst each other by the ROC curve analysis. The model, Fibroscore, with fewer variables and best area under the ROC was then selected. The regression function was:

\[ y = \exp \left[ -4.795 + (0.189 \times \text{bilirubin}) + (0.120 \times \text{GGT}) + (0.080 \times \text{hyaluronic acid}) + (0.518 \times \text{alpha 2 macroglobulin}) - (0.040 \times \text{platelets}) \right] \]

with bilirubin expressed in µmol/L; GGT in U/L; hyaluronic acid in µg/L; alpha 2 macroglobulin in g/L; and platelets in $10^9$/L.

The model predictability was presented as area under the ROC in logistic regression. In addition to the central cut off point of 0.5, various other cut off points were chosen on either side to predict the presence or absence of different stages of fibrosis. The diagnostic values of the model were also presented by the sensitivities, specificities, positive and negative predictive values, and diagnostic accuracy along these cut off points between 0 and 1.
The Fibroscore model was also used to predict moderate to severe necroinflammatory activity from no to mild activity.

The platelet parameters were also assessed in univariate analysis (student t-test) for their association with advanced fibrosis (F3-F4). They were then combined into a simple arithmetic index, **PDW Index**, to predict significant fibrosis.

\[
\text{PDW Index: } \frac{(PDW/\sqrt{MPV})^2 \times 100}{\text{Platelet count}}
\]

It has shown better correlation with significant fibrosis. The PDW Index diagnostic accuracy was then compared with the well known APRI, AST to Platelet Ratio Index, via ROC curve analysis.

The statistical software used was SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).
ANALYSIS
DISCUSSION
Introduction

The present work is an original study for the evaluation of noninvasive markers of liver fibrosis in our country. There has been few local studies describing the role of noninvasive markers, but those have been limited in scope. These studies evaluated only one or two markers. This study is comprehensive, in all 25 markers were studied, includes direct and indirect markers, and evaluates these markers both individually and collectively for the noninvasive evaluation of liver fibrosis. In addition some of the platelet parameters were also assessed and related to the noninvasive evaluation of liver fibrosis.

Due to the limitations and risks of biopsy, as well as the improvements of the diagnostic accuracy of new noninvasive markers, numerous studies strongly suggest that liver biopsy should no longer be considered mandatory as a first line mode of evaluation in some chronic liver diseases.

For example, for the diagnosis of liver fibrosis one of the panels of biomarkers, the Fibrotest has been extensively studied and validated both alone and in combination with elastography or other indices in various algorithms. Between 2001 and 2006, Fibrotest has been assessed in 51 publications; most studies have been performed in hepatitis C (> 5000 patients), in hepatitis B (> 2000 patients), and also in nonalcoholic fatty liver disease, NAFLD, (> 1000 patients).

The practices have been evolving rapidly and a nation wide survey in France found that among 546 hepatologists; 81% used a noninvasive marker (Fibrotest-Actitest), and 32% used elastography with a dramatic decrease in the use of liver biopsy for more than 50% of patients with chronic hepatitis C. A recent overview by the French health authorities officially approved noninvasive biomarkers, Fibrotest, and elastography (Fibroscan) as first line estimates of fibrosis in patients with chronic hepatitis C; recommended reimbursement by social security and approved liver biopsy only as a second line estimate in cases of discordance or non-interpretability of noninvasive biomarkers.
**Fibroscore**

In this study 25 markers were assessed for their association with liver fibrosis. Those markers showing the best association were then entered into stepwise regression analyses to create a predictive model, the (*Fibroscore*). It consists of 5 markers: bilirubin, gamma glutamyl transferase, hyaluronic acid, alpha 2 macroglobulin, and platelets.

The Fibroscore was quite useful in predicting different stages of fibrosis in all patients with chronic viral hepatitis. The Fibroscore values (range 0.00-1.00) increased as the fibrosis stage increased. The maximum score close to 1.00 was for stage 4 (F4, Cirrhosis).

A central cut off of > 0.5 predicted clinically significant fibrosis, CSF, (F2-F3-F4) with a sensitivity of 82% and specificity of 92%. It provided a positive predictive value (PPV) of 79%, and overall diagnostic accuracy of 89%. The same cut off for advanced fibrosis (F3-F4) increased the sensitivity to 85%.

Increasing the cut off value to 0.65 increased the specificity to more than 95% (98%) with a PPV of 95%.

The patients with cirrhosis (F4) had values close to 1.00.

Similarly, the Fibroscore value of < 0.5 provided more than 90% negative predictive value, NPV, for the exclusion of clinically significant fibrosis. The same cut off value has 95% NPV for the exclusion of advanced fibrosis.

Lowering the cut off to < 0.08 for the exclusion of stages F2-F4 provided 98% NPV, thus almost certainly ruling out clinically significant fibrosis.

Thus it can be seen that the Fibroscore is very useful in classifying patients in to various fibrosis categories. This may guide decision to avoid liver biopsy in individual patients, for treatment decisions in patients with chronic HCV (for e.g., genotype 1), and chronic HBV (together with DNA and ALT levels), to continue or otherwise treatment in patients with significant side effects from treatment, for re-treatment of non responder chronic hepatitis patients. A lower Fibroscore values may provide prognostic information in patients who are unlikely to develop liver related morbidity and mortality in the absence of advanced fibrosis. Finally, it may be useful to avoid liver biopsy in patients in
whom occult cirrhosis is suspected and to guide decisions about screening these patients for related complications such as varices.

**Clinical application**

The Fibroscore results can be applied to clinical practice in three ways, analogous to similar panels reported previously:

1. First, it can be applied as a qualitative variable (i.e., indicating the absence [< 0.5] or presence [> 0.5] of CSF). Although this appears a rather simplistic interpretation, the underlying statistical principle (binary logistic regression analysis) between two categorical dependent variables (absence or presence of CSF) has been designed towards this. The Fibroscore values range from 0.00-1.00; a central cut off of 0.5 thus classifies patients on either side, presence or absence of CSF.

2. Second, the probability score can be used as a semiquantitative variable by giving the correspondence between the score and the five fibrosis stages, which is interesting from a clinical point of view, as has been provided by the Fibrotest investigators.

3. Third, the probability score can be used as it is (i.e., as a quantitative variable, such as fibrosis score ranging from 0 [lack of fibrosis] to 1 [cirrhosis]). It is more reliable to use the probability score as a crude measure. Thus for example, a value of 0.48 means no CSF, whereas the fibrosis stage is probably F1. However this probability score is close to the probability threshold of 0.5, thus a value of 0.52 (which is close to 0.48) means that CSF and stage F2 are probable. This example shows that a crude measure (0.48) is a better indicator of the patient’s results than a qualitative interpretation such as no CSF or F1.

However it must be mentioned that a CSF probability score is a fibrosis meter with no unit of reference and with a nonlinear relationship to the F stages.

The noninvasive markers work best at the extremes of fibrosis stages. There are few misclassified patients. What are factors behind misclassification of patients?
Important factors are liver biopsy related, sampling error and interobserver variability in histopathological interpretation.

Another practical difficulty is that of transforming staging into a binary variable when staging is evaluated by the noninvasive tools (the presence or absence of CSF). Whereas staging usually includes five stages from F0 to F4, the cut off is fixed at F2 to define clinically significant fibrosis, CSF, as F2 or higher\textsuperscript{17}.

But more importantly the misclassification relates to the liver histopathology itself. There is considerable overlap between fibrosis scores of F1 and F2 stages. This has been described in the studies before. This may in part reflect the variability in pathologists’ interpretation, with poor inter-observer agreement in the scoring of these stages\textsuperscript{18}.

Furthermore, it has been observed by previous studies that plots of blood tests’ results in relation to F stages, all had same V-shape with a nadir at the F2 stage, suggesting that the difficulty in distinguishing F2 from F1 or F3 stages via histology was the main cause of misclassification\textsuperscript{17}.

Whichever scoring system is used, the histological staging of liver fibrosis on biopsy is artificially represented as a quantitative categorical variable with a progression in severity from 0-4 or 6\textsuperscript{19}. The staging system itself may not reflect a linear increase in fibrosis. In particular, the increase in the degree of fibrosis between F1 (enlarged portal tract) and F2 (enlarged portal tract with rare septae) may not be as great as the increase between F2 and F3 (enlarged portal tract with many septae/bridging fibrosis)\textsuperscript{20}. This does not accurately reflect the dynamic biologic process of fibrosis and constrains the serum marker test performances that are capable of generating continuous variables\textsuperscript{19}.

Indeed, in the early stage disease, there is poor correlation between degree of liver fibrosis as detected by digital image analysis and staging by a pathologist \textsuperscript{21}. Because serum markers are likely to reflect the quantity of fibrotic matrix/tissue, they may correlate better with fibrosis as detected by image analysis than fibrosis stages as determined by the pathologist\textsuperscript{20}. Thus the biochemical markers might represent a more accurate description of fibrogenic events that occur across the liver\textsuperscript{13}.

The quality of this study conformed to the “quality assessment of diagnostic accuracy studies”, (QUADAS), tool \textsuperscript{22}. It is explicit about patient selection and outcomes,
and used an accepted reference standard (rather used the “gold standard” [liver histopathology] for comparison). All patients included in the study received verification using a reference standard (liver biopsy) of diagnosis which was regardless and independent of the index test (noninvasive markers), i.e., the reference standard did not form part of the index test.

This study was conducted in a primary care facility, which is in contrast to other studies carried out in tertiary care centers where relatively sick patients were evaluated (with over representation of advanced disease). Thus the spectrum of patients would be the same who will receive the test in practice (i.e., a community based practice); where the prevalence of the disease in the population being studied is closer to the general population at large. All of these factors have been cited as the most important criteria that impact on study quality 19.

Additionally, in the context of this particular study, the pros and cons of reference standard are described in detail, the composition of panel of serum markers reported and the formula derived for the panel of serum markers 19.

**Importance of Direct and Indirect Markers**

Broadly there are two types of noninvasive markers, indirect and direct markers. Each particular type has its merits. For instance, the indirect markers are easily accessible 13,17,20,23-25, but on the other hand, the direct markers are known for their putative specificity 26-29. It has been proved by various studies that the combination of both types of markers may increase certain advantages and limit other disadvantages 30-32.

The direct markers are more specific but are readily altered by other phenomenon such as connective tissue damage 17. Indeed, their relationship with fibrosis is complex. One explanation is that, the blood markers interact both with the dynamics and the cause of fibrosis 33,34. For example, hyaluronic acid (HA) is a marker of fibrogenesis 35, and its blood level also depends on the persistence of the cause of chronic liver disease (CLD) 36. Thus the exact meaning of these tests requires further investigation 17.

Indeed, some of the previous studies have limited the inclusion of markers to one of the two categories. Thus according to one source 17, Rosenberg et al 26, stated that the
inclusion of Prothrombin index (PI, the prothrombin time is expressed as percentage of the standard value) and the platelet count didn’t increase the performance of direct markers

Isolated markers have smaller diagnostic value than panels of noninvasive markers\textsuperscript{11,27}. Individually the markers are useful in the assessment of liver fibrosis, but collectively they add more information to the diagnostic ability. The combination of markers slightly increases sensitivity, specificity, negative and positive predictive values presumably due to isolated variations in biomarkers for diseases other than liver fibrosis\textsuperscript{17}. Also in statistical analysis, by combining these markers (independent variables) into a regression model, their collective contribution to the model (correlation with the dependent variable [fibrosis on liver histopathology]) may be more than their individual association (correlation) with the dependent variable\textsuperscript{37}.

**Noninvasive Markers in the Study**

In this study, age and different markers that have shown association with fibrosis include: bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), cholesterol, prothrombin time, albumin, immunoglobulin G (IgG), urea, hyaluronin acid (HA), alpha 2 macroglobulin (A2M), platelet count, mean platelet volume (MPV), and platelet distribution width (PDW). All these factors either reflect liver function and/or are fibrosis markers.

In the present study, age has not come up as a major factor influencing fibrosis, as has been in some of the other studies, presumably due to certain demographic circumstances, a relatively younger population was studied. Similarly the relative percentage of patients in different stages of fibrosis is also variable.

Age at infection has been shown to influence the outcome of hepatitis C and patients infected after the fourth decade have a higher risk of disease progression\textsuperscript{38-40}. It is evident that the duration of the hepatitis infection would be a more precise indicator of fibrosis than age, as also evidenced by our study, in which some of the relatively younger population has evidence of advanced fibrosis. It has been previously mentioned in the literature that the precise timing and the mechanism of acquisition of hepatitis infection
remains largely unknown in most of the patients, therefore the duration of hepatitis infection is difficult to establish \(^23\).

As fibrosis progresses the concentration of bilirubin increases as a result of reduced hepatic excretion and less enterohepatic circulation attributable to portal systemic shunting \(^41\).

ALT is the most commonly investigated biomarker, but with a low sensitivity of 61-71\%. This diagnostic value is lower than the combination of markers in all direct comparisons. However, it is an invaluable component of the grade of necroinflammatory activity as evidenced by its inclusion in the Actitest (a modification of the Fibrotest that incorporates ALT in addition to other 5 parameters) \(^10\).

AST is another commonly investigated marker with many studies either in isolation or more commonly with other parameters \(^24\). It is thought that the progression of liver fibrosis may reduce clearance of AST \(^42\), leading to increased serum AST levels. In addition advanced liver disease may be associated with mitochondrial injury, resulting in more marked increase of AST, which is present in mitochondria and cytoplasm, relative to ALT \(^24,43\). In the present study, although AST has shown correlation with fibrosis, there were other markers which had shown stronger association. It may be that AST levels significantly increase in advanced liver disease.

GGT has been strongly correlated with liver fibrosis among patients with hepatitis B and C \(^13,20,23,44\). Early steatosis or an increase in epidermal growth factor could be one explanation for the GGT increase with the severity of fibrosis \(^45\). In one of the studies it was shown that in patients with chronic hepatitis C bile duct damage was present in more than one third of patients with substantial fibrosis (stages 2-4) in comparison with less than 10\% in patients with fibrosis stage 0-1. In this study GGT was an independent predictor of bile duct damage \(^46\).

Albumin had no independent diagnostic value, most likely because patients with advanced cirrhosis were excluded \(^13\). Urea synthesis is also decreased in patients with cirrhosis \(^47\).

IgG (\(\gamma\)-globulin) concentration is associated with cirrhosis and portosystemic shunts \(^48\). Serum immunoglobulins are produced by B lymphocytes and thus measurement of these substances is not a direct test of liver function. Earlier on it has
been believed that, elevation of serum immunoglobulin levels in many patients with chronic liver disease is believed to indicate impaired function of reticuloendothelial cells in hepatic sinusoids or shunting of portal venous blood around the liver. Data indicate that antibodies directed against antigens of the normal colonic flora account for much of the increased serum immunoglobulin levels in patients with cirrhosis. In cirrhosis, these antigens are not taken up and degraded by the hepatic reticuloendothelial cells as they are normally, and reach the lymphoid tissue outside the liver, where they elicit an inflammatory response.

Studies indicate a strong association between serum immunoglobulin levels (IgA, IgG and total) and hepatic fibrosis in patients with HCV infection; and between levels of serum globulin and IgG and extent of hepatic fibrosis in patients with chronic HBV infection. For each increase of 0.33 mg/dL in serum globulin, there was a 0.5 point increase in the stage of hepatic fibrosis in chronic HBV infection.

It has been postulated that the removal of immunoglobulins by the liver may be impaired in patients with severe liver dysfunction because the liver is a major catabolic site for immunoglobulins; possibly by a deficient receptor-mediated mechanism.

In addition, in recent years several studies have reported that platelet associated immunoglobulin G levels are elevated in patients with chronic hepatitis and cirrhosis and have a role in immune mediated thrombocytopenia in these patients.

The relationship between cholesterol and fibrosis has been investigated before. The decrease in cholesterol levels seen in patients with advanced cirrhosis is caused by a reduction in cholesterol synthesis, and, therefore it is unlikely that the synthetic capacity of hepatocytes can be altered before liver cirrhosis is established.

HCV infection is associated with clinically significant lower cholesterol levels (TC, LDL and HDL) when compared with those of normal subjects. This finding is more pronounced in patients infected with HCV genotype 3a. In our study HDL, and TG were significantly decreased in patients with HCV, as compared to HBV, while total cholesterol and LDL levels had minimal difference.

Hepatocellular steatosis is common in patients with chronic hepatitis C. Steatosis can be considered as a true cytopathic lesion induced by hepatitis C virus (HCV) genotype 3. The authors in one study found that HCV core protein-lipid droplet
interaction could play a role in virus-induced steatosis. But importantly, they found no genetic or functional differences between genotype 3a core proteins from patients with and without HCV-induced steatosis. Thus the investigators concluding that other viral proteins and/or host factors are involved in the development of hepatocellular steatosis in patients infected by HCV genotype 3a. In our study a significant proportion of patients with HCV had hepatic steatosis as compared to HBV.

Hyaluronic Acid is a high molecular weight glycosaminoglycan, which is an essential component of extracellular matrix in virtually every tissue in the body.

In patients with chronic hepatitis C virus, HA levels increase with the development of liver fibrosis. Moreover, in patients with cirrhosis, HA levels correlate with clinical severity. Liver injury increases hyaluronic acid (a glycosaminoglycan) production by the hepatic stellate cells and decreases its clearance by the sinusoidal endothelial cells. These cells degrade hyaluronic acid in a specific receptor mediated process and its elevated levels in cirrhosis are presumed because of sinusoidal capillerisation.

In one of the recent studies, direct quantitative association was observed between HA level and hepatic hydroxyproline content in a cirrhotic rat model. Of all the direct markers, HA has shown the strongest association with liver fibrosis.

Alpha 2 macroglobulin is another very important marker and has shown strong association with liver fibrosis. Hardly known outside the research realms, it became widely known after the noninvasive test panels became popular. Initially a significant diagnostic value of increased alpha 2 macroglobulin was noted for fibrosis staging in patients with alcohol liver disease. It is a protease inhibitor whose concentration increases with stellate cell activation and liver fibrosis. It is also an acute phase protein and is produced at sites of inflammation and liver fibrosis by hepatocytes, stellate cells and granuloma cells.

Alpha 2 macroglobulin is related to fibrosis since it is a feature of stellate cell activation. Being a proteinase inhibitor, its increased synthesis can inhibit catabolism of matrix proteins and enhance fibrotic processes in the liver. As seen in experimental fibrosis, an increase in hepatocyte growth factor could account for the unexpected fall in reduction in transforming growth factor-β1 (TGF-β1), rise of A2M, and decrease of...
haptoglobin. Transduction with hepatocyte growth factor gene suppresses increase of TGF-β1 \(^{70}\) and the factor stimulates synthesis of A2M \(^{13,71}\).

Unlike hyaluronic acid, which has stronger correlation with higher grades of liver fibrosis, alpha 2 macroglobulin shows association with liver fibrosis across all stages. In the original study by Imbert-Bismut et al, (Fibrotest), of all the markers, alpha 2 macroglobulin was the most informative \(^{13}\). Alpha 2 macroglobulin has been the component of all the major test panels of noninvasive markers of liver fibrosis \(^{13,17,20,27,28}\).

**Platelet Parameters, PDW Index, and Liver Fibrosis**

The platelet parameters that have shown association with liver fibrosis include: platelet count, Platelet distribution Width (PDW); and these together with Mean Platelet Volume (MPV) were combined into an index, the PDW index.

For the prediction of advanced fibrosis (F3, F4), at a cut off of 7.00, the PDW index had a sensitivity of 75%, specificity of 91%, and overall diagnostic accuracy of 87%. And for the prediction of advanced fibrosis, the PDW ROC was 0.840, while that of APRI was 0.888. The ROC’s for APRI are similar to the original study by Wai, et al. Thus it can be seen that the ROC of PDW Index is comparable to APRI for the prediction of advanced fibrosis.

Thrombocytopenia is a commonly encountered hematological condition in chronic liver disease and cirrhosis \(^{54}\). Historically, it has been attributed to the sequestration and destruction of platelets in the enlarged spleen with an impaired ability of bone marrow to compensate by increasing platelet production \(^{72,73}\). However attempts to correct the low platelet levels by splenectomy and portal decompression procedures have failed to consistently improve the platelet count in the long term \(^{72,74}\).

A study has shown that in patients without splenomegaly, the thrombocytopenia was associated with the stage of fibrosis; platelets counts were the highest in patients with fibrosis stage 0-2 (Knodell HAI), lower in those with stage 3 (p< 0.008) and lowest in those with stage 4 (p< 0.05) \(^{75}\).

It has been postulated that auto-antibody mediated platelet destruction, as seen in patients with idiopathic thrombocytopenia purpura (ITP) may also contribute to cirrhotic
thrombocytopenia. In this situation, the thrombocytopenia is principally mediated by enhanced platelet clearance in the periphery, resulting in accelerated platelet turnover. Additionally, impaired platelet production due to thrombopoietin (TPO) deficiency has also been proposed as another cause of thrombocytopenia in cirrhosis patients. However, several other studies have found that the circulating TPO level is maintained or even increased in patients with cirrhosis.

Thus according to one review, in patients with chronic liver disease (CLD) from HCV, and also in patients with CLD in general, both prevalence and severity of thrombocytopenia increase in parallel with the extent of disease, usually becoming clinically relevant when patients develop extensive fibrosis and/or cirrhosis.

Pathogenetic mechanisms for thrombocytopenia in advanced liver disease include hypersplenism secondary to portal hypertension, bone marrow suppression (resulting in suppression of megakaryocytes) likely by the disease itself, and the use of drugs, and aberrations of the immune system resulting in the formation of anti-platelet antibodies and/or immune-complexes that bind to platelets and facilitate their premature clearance. In chronic liver disease, the natural inverse relationship between TPO and platelet levels is not maintained; therefore, blood TPO levels fail to have clinical relevance or predictive value in assessing the thrombocytopenic status of a given patient.

The quantitation of platelets in peripheral blood is a well-recognized tool. Recently, new indices related to platelet counts have been provided by hematologic analyzers. The important parameters are mean platelet volume (MPV), and platelet distribution width (PDW). Though routinely evaluated, the clinical inferences are usually not drawn.

A decrease of mean platelet volume (MPV) has been observed in liver cirrhosis with thrombocytopenia. It has also been shown that, platelet distribution width is a better indicator of altered platelet homeostasis than MPV in liver cirrhosis. PDW is markedly increased in cirrhosis as compared to controls, while MPV was not significantly different.

One study reported that, in the diagnosis of thrombocytopenia in aplastic anemia (AA) and immune thrombocytopenia (ITP); platelet-large cell ratio (P-LCR), and platelet size deviation width (PDW), had the larger area under the ROC, than mean platelet
volume (MPV). This indicated that these values were very reliable for immune thrombocytopenia. Thus there is increase in PDW in auto-antibody mediated platelet destruction in patients with idiopathic thrombocytopenia purpura (ITP).

In cirrhotic thrombocytopenia, one of the contributory factors is the auto-antibody mediated platelet destruction, like ITP, as already discussed\textsuperscript{54}. Thus this mechanism may also contribute to the increase in PDW seen in patients with advanced liver disease.

**Important studies of Noninvasive Evaluation of Liver Fibrosis**

In the past few years the noninvasive evaluation of liver fibrosis has been an active area of research and investigation.

Of the noninvasive marker panels, **Fibrotest\textsuperscript{13}** is the most well known and validated of all with more than 50 studies to its credit\textsuperscript{10}. The 5 indices of the panel are: total bilirubin, GGT, haptoglobin, alpha 2 macroglobulin and apolipoprotein A1; these are entered with the patient’s age and gender in a patented artificial intelligence algorithm (USPTO 6631330) to generate a measure of fibrosis stage\textsuperscript{10}.

**Actitest** is a modification of Fibrotest that scores necroinflammatory activity (Grade in liver histology), and incorporates ALT in addition to the five markers. In addition to the Actitest, the other panels are: Steatotest, for the quantitative assessment of steatosis; Nashtest, for the categorical assessment of nonalcoholic steatohepatitis; and Ashtest, for the quantitative assessment of alcoholic steatohepatitis. The last three panels combine all the six components of Fibrotest-Actitest and, AST, fasting glucose, total cholesterol, triglycerides, height, weight (BMI), age and sex of the patient\textsuperscript{10}.

All the above mentioned panels are combined into a supercombination, **FibroMAX**, in patients at risk of chronic liver diseases\textsuperscript{10}. The Fibrotest-Actitest provide a continuous linear estimate ranging from 0.00 to 1.00 corresponding to the well established Metavir scoring system of stages from F 0-4 and Grade A 0-3\textsuperscript{10}.

For Fibrotest, the area under the receiver operating characteristic curve, ROC, was 0.836 to 0.87 in the principal study\textsuperscript{13}. In subsequent studies the ROC curves have varied from 0.65 to 0.89. This could be attributed to the variations in the study protocols.
analytical variability of the tested markers, and variability of fibrosis stage prevalence defining advanced and non-advanced fibrosis\textsuperscript{10,81}.

With the best index, a high negative predictive value (100\% certainty of absence of F2, F3, or F4 fibrosis) was obtained in scores ranging from zero to 0.10 (12\% of all patients), and high positive predictive value (>90\% certainty of presence of F2, F3, or F4 fibrosis) for scores ranging from 0.60 to 1.00 (34\% of all patients). The detection of significant fibrosis F2 or greater had a 75\% sensitivity and 85\% specificity. The assay performed somewhat better for the assessment of more advanced liver disease (METAVIR stages 3 and 4)\textsuperscript{13}.

The discordances between the Fibrotest and biopsy are similar. According to one study 18\% of the discordances were attributable to biopsy failure (mostly due to short length) and 2\% to Fibrotest\textsuperscript{82}. In another prospective multicenter study, the Fibropaca study, the discordance was attributable to biopsy in 3.8\%, to Fibrotest in 5.4\%, and undetermined in 9.1\%\textsuperscript{31}.

The Fibrotest scores fall in response to therapy. Patients who had a sustained virological response to interferon had a substantial reduction in Fibrotest and Acitext scores, as compared to those who were either primary nonresponders or relapsed following therapy\textsuperscript{83}.

The 5 year prognostic value of Fibrotest is at least equal to liver biopsy\textsuperscript{84}.

In addition to analytic variability (discussed below), there are issues related to the false positive/negative values of Fibrotest components, that could artificially increase/decrease the suspicion of liver fibrosis. The most frequent abnormal value of Fibrotest markers noted in the general population, since its inception is low haptoglobin\textsuperscript{10}. Extremely low haptoglobin, especially when other exams are hardly modified could have hemolysis. False positives due to increase in bilirubin, most likely due to Gilbert’s syndrome have also been observed, although hemolysis is a second thought. Acute sepsis/inflammation can also raise alpha 2 macroglobulin and haptoglobin levels. If these conditions are suspected, the Fibrotest evaluation should be postponed\textsuperscript{10}. 

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There has been concern about the analytic variability of proteins of Fibrotest components.

In this regard, a study compared the variability of Fibrotest proteins (including alpha 2 macroglobulin) using two different nephelometric analyzers: the BN Prospec ® (Dade-Berhing) i.e., the technique used by the original reference laboratory, and the Immage ® (Beckman-Coulter) another widely distributed nephelometric instrument. For both analyzers the reagents were standardized against the International Certified material 470 for alpha 2 macroglobulin 85.

Levels of alpha 2 macroglobulin measured with Immage ® analyzer were significantly higher than those with BN Prospec ® analyzer. The median difference between the results obtained with the two analyzers was 20.5% (range 4.8-32.3) 86.

Subsequently, the same group, however, in another study, for analytic variability for Fibrotest components, used new reagents for alpha 2 macroglobulin and found excellent transferability between the Immage ® and the BN Prospec ® analyzers, with fewer than 10% of the values outside the accepted boundaries.

The reason for this dramatic improvement was that in the subsequent study, a new antibody was used in the Beckman-Coulter reagent (the analyzer different from the Fibrotest reference laboratory analyzer) 87.

The authors suggesting that in addition to the antibody, other factors could cause variations, such as: the calibrator and the type of calibration, antigen excess, matrix differences, molecular weight and/or the functional conformation of the proteins, length of the immunological reaction, and finally individual genetic heterogeneity 87.

Another very widely reported index, APRI refers to the AST to Platelet ratio index. It has been assessed in several studies and has shown rather good diagnostic performance and reproducibility, particularly for cirrhosis ( ROC between 0.77-0.94). In the original study by Wai et al, the model predicted bridging fibrosis with a ROC of 0.80-0.88 24. APRI has lower accuracy (ROC of 0.74) for detecting lower grades of fibrosis 88.

Its most important aspect is the use objective and readily available laboratory values. Both platelet count and AST levels are routine tests performed in
chronic hepatitis C patients in clinical practice, so no additional tests are required Though slightly inferior, nevertheless, its simplicity (APRI can be determined by the bedside with the help of a calculator), matched with performance to the more sophisticated Fibrotest and Forn’s index is a great advantage.

The Forn’s index is based on four readily available variables; age, platelet count, γ glutamyl transferase, and cholesterol levels. The area under the ROC curve was between 0.81 - 0.86 for the estimation group and the validation groups. The lower cut of value had a 96% negative predictive value, whereas the upper cut off value had a positive predictive value of 66%. Hence this test was useful at excluding patients with minimal fibrosis, but was of limited value for the identification of patients with more advanced liver disease.

One important aspect of the index is the use of very basic clinical parameters, and the fact that the accuracy of this model compares with models based on more sophisticated variables (such as Fibrotest). Major caveats of the Forns index include concerns about the impact of lipid abnormalities in patients with hepatitis C, cholesterol altering medicines, and the reproducibility of platelet estimations.

The Sud score incorporated age, past alcohol intake, AST, cholesterol, and insulin resistance had a ROC of 0.77.

The Europeon Liver Fibrosis Group study (ELFG) by Rosenberg et al, evaluated an algorithm consisting of age, hyaluronic acid, tissue inhibitor of metalloproteinase 1 (TIMP-1), and N-terminal propeptide of type III collagen (PIIINP). It had a ROC of 0.78 for significant fibrosis from all etiologies, and a ROC of 0.77 when limited to patients with hepatitis C.

In a recent study, in which hyaluronic acid (HA) was compared with Fibrospect II (consisting of HA, TIMP-1, and alpha 2 macroglobulin), and YKL-40, concluded that: HA was effective in discriminating between significant fibrosis as compared to FS II with a ROC of 0.76 vs 0.66.

Hepascore another model consisting of bilirubin, GGT, hyaluronic acid, alpha 2 macroglobulin, age and sex had area under the ROC of 0.85 for significant fibrosis,
which increased to 0.96 and 0.94 for advanced fibrosis and cirrhosis respectively. The Hepascore increased significantly (p<0.001) as the fibrosis stage increased. A central cut off point of 0.5 predicted significant fibrosis with a sensitivity of 67% and a specificity of 92%. When the same cut off point of 0.5 was applied for the prediction of advanced fibrosis (F3-F4) sensitivity was 95% and specificity was 81%. A cut off point of 0.84, when applied for the detection of cirrhosis (F4) provided a 71% sensitivity and 84% specificity.

The Fibrometer test, developed by Calès et al, combined platelets, prothrombin index, AST, alpha 2 macroglobulin, hyaluronic acid, urea and age. Its area under ROC in patients with viral hepatitis for significant fibrosis (stage F2-4) was 0.883 compared with 0.808 for the Fibrotest (p = 0.01), 0.820 for the Forns test (p = 0.005), and 0.794 for the APRI test (p < 10^-4). The Fibrometer area under ROC curve increased to 0.892 in the validating population.

The authors also estimated the area of fibrosis in viral hepatitis by testing for hyaluronate, γ glutamyl transferase, bilirubin, platelets, and apolipoprotein A1. The use of blood tests to estimate the area of fibrosis (AOF) is new. Measurement of area of fibrosis via image analysis is limited to clinical research. Unlike histological stages, the AOF provides precise quantification of the extensive variations in fibrosis during cirrhosis. Because cirrhosis corresponds to only one or two stages the range of blood test results for clinically significant fibrosis (CSF) in patients with cirrhosis is very limited.

Thus in viral related chronic liver disease, the interquartile range of blood tests for AOF was 7.2% to 10.4% in F0-F3 versus 11.2% to 19.0% in F4; whereas the corresponding ranges for the Fibrometer test for CSF were 0.03 to 0.99 and 0.99 to 1.00, respectively. Moreover, the AOF estimation via blood test is the only statistically validated quantitative test for the noninvasive diagnosis of fibrosis.
Genetics and Proteomics

In addition to the indirect and direct noninvasive markers, other novel and exciting new indices have been developed.

The complexity of the fibrogenetic process and the high number of factors/cytokines/molecules involved imply that several genetic polymorphisms could influence progression of liver fibrosis. There are many studies that report gene polymorphisms to either favor or reduce fibrogenesis in patients with different forms of chronic liver disease. While these studies clearly indicate that many genetic factors have a definitive influence on the risk of developing a more or less active and progressive fibrogenesis, very few of them have found an application as a diagnostic/prognostic marker in clinical practice, due to their complexity, difficulty to test and variable behavior in different patient populations.

The clinical factors such as age, gender, alcohol use, and age at infection, obesity and hepatic steatosis, influence the progression to cirrhosis, but cannot accurately predict the risk of developing cirrhosis in patients with chronic hepatitis C. One such study, consists of a set of seven marker genes, the cirrhosis risk score (CRS), was found to be a better predictor of high risk versus low risk for cirrhosis in Caucasian patients than clinical factors. The authors validated all significant markers from a genome scan in the training cohort, and selected 361 markers for the signature building. Subsequently, a signature consisting of 7 markers most predictive for cirrhosis risk in Caucasian patients was developed.

The area under the receiver operating characteristic (ROC) curve was 0.75 in the training cohort. In the validation cohort, the ROC was only 0.53 for clinical factors, increased to 0.73 for CRS, and 0.76 when CRS and clinical factors were combined. The authors concluding that, CRS is a better predictor than clinical factors in differentiating high risk versus low risk for cirrhosis in Caucasian patients.

A study using profiles of serum protein N-glycans found that a profile has a similar area under ROC to Fibrotest for the diagnosis of compensated cirrhosis. When
compared with Fibrotest, this marker had 100% specificity and 75% sensitivity for the
diagnosis of compensated cirrhosis, which is not greatly different from the 92%
specificity and 67% sensitivity of the Fibrotest alone.\textsuperscript{96}

Proteomics studies are ongoing, and the components of the Fibrotest have been
identified in several proteomic studies.\textsuperscript{97,98} A combination of proteomic peaks has been
identified with a 7% increase in area under the ROC in comparison with the Fibrotest.\textsuperscript{10} Even if the diagnostic value of these panels is significantly greater than the reference
panels, the cost utility and the availability to the population at large are important
concerns.

\textbf{Elastography}

Another noninvasive method for the assessment of liver fibrosis is elastography
(FibroScan). In a study by Castera et al, the area under the ROC by FibroScan was 0.83
for F\textless 2, 0.90 for F\textless 3, and 0.95 for F=4. The same authors demonstrated that, when
combined with Fibrotest, FibroScan was more precise than liver biopsy, the area under
the ROC reaching 0.88, 0.95 and 0.95 for fibrosis stages F\textless 2, F\textless 3 or F4 respectively.\textsuperscript{14}

In the study performed by Ziol et al, the area under the ROC value of FibroScan
as compared to liver biopsy was 0.79 for F\textless 2, 0.91 for F\textless 3, and 0.97 for F= 4. The
authors concluding that elastography appears reliable to detect significant fibrosis and
cirrhosis in patients with chronic hepatitis C.\textsuperscript{99}

In one of the metanalysis, for significant fibrosis, the area under the ROC for
Fibrotest and FibroScan were 0.81 (95% CI 0.78-84) and 0.83 (0.03-1.00), respectively. At a threshold of approximately 0.60, the sensitivity and specificity of the Fibrotest were
47% (35-59%) and 90% (87-92%). For FibroScan (threshold approximately 8 kPa),
corresponding values were 64% (50-76%) and 87% (80-91%), respectively. For cirrhosis,
the summary area under the ROC for Fibrotest and FibroScan were 0.90 (95% CI not
calculable) and 0.95 (0.87-0.99), respectively. The diagnostic accuracy of both measures
was associated with the prevalence of significant fibrosis and cirrhosis in the study
populations.\textsuperscript{100}
In one of the studies, in patients with HCV infection, the sensitivity of Fibrotest appears higher for early stage fibrosis, and the combination of FibroScan and Fibrotest improves the overall diagnostic value. Elastography is complementary because of its specificity for severe fibrosis, and also in case of Fibrotest, some patients with high risk profile of false positives and negatives\textsuperscript{15}.

\textit{It is probable that in not too distant future, the combination of Fibrotest (or other combination of noninvasive biomarkers) with FibroScan could replace liver biopsy in most patients with chronic hepatitis}\textsuperscript{14,101}. 
REFERENCES


RESULTS
Patient Characteristics

The clinical and laboratory characteristics of patients are presented in the following Tables. A total of 100 patients were enrolled in the study, of which 88 were included in the final analysis. Their mean age was 32.43 years (range 20-53 yrs). There were 41 (46.6%) females. There were 74 (84%) patients with chronic liver disease from HCV, 13 (15%) with HBV, and 1(1%) patient had both HCV RNA PCR and HBsAg positive. The clinically significant fibrosis (CSF) as defined as stages 2, 3 and 4 (F2, F3, F4) was present in 23 (26%) patients; and advanced fibrosis, stages 3 & 4 (F3-F4) was present in 20 (23 %) of patients, which generally is regarded as the prevalence in the community. The mean length of the biopsy specimen was 1.15 cm (median 1.00 cm), and the interobserver agreement (Kappa, κ coefficient) between the histopathologists was moderate (κ = 0.60) for grading and good for staging (κ = 0.74).

Predictive Model (FIBROSCORE)

Various markers were assessed for their association with clinically significant fibrosis Table. The most useful variables were then entered into step wise logistic regression function to create different probability models. The best model with the largest area under the receiver operating characteristic curve (ROC) with the fewer variables was selected.

This model Fibroscore consisted of 5 markers: bilirubin, gamma glutamyl transferase (GGT), hyaluronic acid (HA), alpha 2 macroglobulin (A2M), and platelets.

The area under the ROC for F2 (stage 2) fibrosis was 0.808 (95% CI; .613-1.00), for F3 the ROC was 0.938 (95% CI; .888-.988), and for F4 the ROC was 0.959 (.893-1.00) and for combined stages 2 and 3 (F2, F3) the ROC was 0.948 (95% CI .902-.994). The overall diagnostic accuracy of the model for predicting clinically significant fibrosis was 89%.

The Fibroscore values (range 0.00-1.00) increased as the fibrosis stage increased (graph 3). The maximum score was for stage 4 (F4). The small box plot for F4 indicates that the corresponding range for the Fibroscore for cirrhosis (F4) is small i.e., 0.99-1.00.
A central cut off of 0.5 was chosen to observe the significance for clinically significant fibrosis. A score of > 0.5 was seen in 24 of 88 (27%) of patients. This central cut off point in the model predicted clinically significant fibrosis (F2, F3 and F4) with a sensitivity of 82% (95% CI; 63-93), specificity of 92% (95% CI; 83-96), positive predictive value (PPV) of 79 % (95% CI; 59-90), negative predictive value (NPV) of 93% (95% CI; 85-97) and overall diagnostic accuracy of 89% (95% CI; 81-94).

Thus 19 of 24 patients (79%) had clinically significant fibrosis (The PPV). Of the 5 misclassified patients with values > 0.5, four patients had moderate activity (ALT range 58-139), and portal fibrosis (necroinflammatory grade in two patients, 8/18; and in two patients, 7/18; fibrosis stage in all four patients 1/4). Only one patient had minimal activity and no fibrosis (1/18, 1/22). Of the 5 misclassified patients with values < 0.5 only two had fibrosis stage 3/4, one had stage 3/6, the other 2/6.

By applying the same cut off point for advanced fibrosis (F3, F4), the sensitivity was 85% (95% CI; 64-94), specificity was 89% (95% CI; 80-94) and overall diagnostic accuracy was about the same, 88% (95% CI; 80-93).

Increasing the cut off point to > 0.65 for clinically significant fibrosis increased the specificity to 98% with a positive predictive value of 95%. Thus 19 of 20 (95%), patients who had Fibroscore value more than 0.65, had clinically significant fibrosis (The PPV). The one misclassified patient had high ALT 139 U/L, moderate activity (8/18), and fibrosis stage 1 on liver biopsy.

Further increasing the cut off to 0.80 increased the positive predictive value to 100%, but only 15 patients out of 88 could be classified (17%).

A score of < 0.5 was observed in 64 (73%) patients, which excluded advanced fibrosis (F3, F4) with a sensitivity of 89%, and specificity of 85%.

The Fibroscore reliably detects the absence of fibrosis. Lowering the cut off to 0.20 reliably predicts the absence of clinically significant fibrosis with a NPV of 96%. Only 2 patients had score less than 0.20, none of these had established bridging fibrosis; one had stage 3/6, other stage 2/6.

Almost 100% certainty of the absence of clinically significant fibrosis (actual NPV 98% of the absence of F2,F3,F4) was predicted by scores between 0.00 to 0.08. The
only one misclassified patient had mild activity and a lower stage of fibrosis (grade 6/18, and stage 2/6).

The Fibroscore was also very useful in differentiating moderate to severe from minimal to mild necroinflammatory activity. The area under the ROC curve was 0.790 (95% CI; .696-.884), with the sensitivity of 63% and specificity of 77%.

**Platelet Variables**

The different platelet variables evaluated in the study for advanced fibrosis stages 3 and 4 (F3-F4) were: platelet count, mean platelet volume (MPV), platelet distribution width (PDW), and giant platelet percentage. Their association with the advanced fibrosis is shown in the Table 28. The covariates that showed statistical significance included: platelet count (F0-F2, mean 266 x 10^9/l, F3-F4 mean 193 x 10^9/l, p = 0.000); PDW (F0-F2, mean 12.37, F3-F4 mean 13.66, p = 0.004; and the PDW index described below (F0-F2 mean 5.7, F3-F4 mean 9.5 p = 0.000).

Of the other parameters, MPV had shown some association (F0-F2 mean 10.66 fl, F3-F4 mean 11.26 fl, p = 0.76), though not statistically significant. In the study giant platelet percentage did not show any association with advanced levels of fibrosis. Apart from the later, the other parameters were combined into an index that had shown good predictability with advanced fibrosis.

**PDW Index**

The platelet count, mean platelet volume (MPV), and platelet distribution width (PDW) were combined into different indices for the prediction of advanced fibrosis (F3,F4); the one with the best association and the best ROC, was derived arithmetically as PDW index.

The area under the ROC for advanced fibrosis (F3-F4) for PDW index was 0.840 (95% CI; .721-.958). The ROC for the prediction of stage 3 (F3) fibrosis was 0.801 (95% CI; .671-.930). For the prediction of advanced fibrosis (F3, F4), at a cut off of 7.00, the index had a sensitivity of 75%, specificity of 91%, PPV of 71%, NPV of 92%, and overall diagnostic accuracy of 87%.
Out of 21 patients with scores more than 7.00, six patients were misclassified. Out of these 6 patients, 4 patients had evidence of moderate activity, the other two had mild activity; all patients had stage 1 fibrosis.

PDW Index performance for the lower grades of fibrosis falls, as for F2, the PDW ROC was 0.635 (95% CI: .521-.749).

In this study, the PDW ROC for the prediction of F3 fibrosis was 0.801, while the corresponding ROC for AST to Platelet ratio index (APRI) for F3, also evaluated in this study, was 0.854. And for the prediction of F3-F4 fibrosis, the PDW ROC was 0.840, while that of APRI was 0.888. The ROC’s for APRI are comparable to the original study by Wai, et al. Thus, it can be seen that the ROC of PDW index has only slight difference at the second decimal point as compared to the ROC of APRI.

Thus it is concluded that the PDW index compares with the renowned noninvasive index, APRI, for the prediction of significant fibrosis. It’s most important aspect is that, it does not use any biochemical markers, and is only derived from platelet parameters, which are generally considered not of great clinical significance.

Additionally, in the context of our country with limited resources, it is very helpful to have an index being derived from blood complete picture only, and unlike the well known index, APRI, by not even using a single biochemical test. Thus as a screening test it may guide towards the use of more expensive tests.

Though somewhat similar in performance (the difference is manifest at the second decimal point), its simplicity (like APRI, PDW index can be determined by the bedside with the help of a simple calculator), matched with performance to the more sophisticated Fibrotest; and the liver fibrosis index, (Fibroscore), evaluated in this study, is a great advantage.
Fig 17. The Fibroscore assessment for clinically significant fibrosis
Table 18. Diagnostic indices of FIBROSCORE > 0.5 for clinically significant fibrosis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Lower – Upper 95 % CIs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>82.61%</td>
<td>(62.86 – 93.02)</td>
</tr>
<tr>
<td>Specificity</td>
<td>92.31%</td>
<td>(83.22 – 96.67)</td>
</tr>
<tr>
<td>PPV</td>
<td>79.17%</td>
<td>(59.53 – 90.76)</td>
</tr>
<tr>
<td>NPV</td>
<td>93.75%</td>
<td>(85.00 – 97.54)</td>
</tr>
<tr>
<td>Diagnostic Accuracy</td>
<td>89.77%</td>
<td>(81.69 – 94.53)</td>
</tr>
</tbody>
</table>
Table 19. Clinical and demographic data of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD)(Range) /Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>32.43 ± 6.1 (20-53) Median 32</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>41 (46.6%)</td>
</tr>
<tr>
<td>Male</td>
<td>47 (52.4%)</td>
</tr>
<tr>
<td>Mode of Transmission</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>54 (61.4%)</td>
</tr>
<tr>
<td>Injections (excluding procedure)</td>
<td>5 (5.7%)</td>
</tr>
<tr>
<td>Transfusions</td>
<td>3 (3.4%)</td>
</tr>
<tr>
<td>Minor Surgery</td>
<td>15 (17%)</td>
</tr>
<tr>
<td>Major Surgery</td>
<td>11 (12.5%)</td>
</tr>
<tr>
<td>BMI</td>
<td>23.9 ± 3.64 (17-35) Median 24</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>22 (25%)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>6 (6.8%)</td>
</tr>
<tr>
<td>HCV RNA</td>
<td>74 (84%)</td>
</tr>
<tr>
<td>HBsAg</td>
<td>13 (14.7%)</td>
</tr>
<tr>
<td>Both</td>
<td>1 (1.13%)</td>
</tr>
<tr>
<td>Knodell HAI</td>
<td>82 (93%)</td>
</tr>
<tr>
<td>Modified Knodell (Ishak)</td>
<td>6 (7%)</td>
</tr>
<tr>
<td>Fibrosis score</td>
<td>1.21 ± 1.14 (0-4), Median 1.00</td>
</tr>
<tr>
<td>Significant Fibrosis (F2-F4)</td>
<td>23 (26%)</td>
</tr>
<tr>
<td>Advanced Fibrosis (F3-F4)</td>
<td>20 (23%)</td>
</tr>
<tr>
<td>Advanced Activity (Mod-Severe)</td>
<td>44 (50%)</td>
</tr>
<tr>
<td>Test</td>
<td>Count</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>HCV RNA PCR</td>
<td>74</td>
</tr>
<tr>
<td>HBsAg</td>
<td>13</td>
</tr>
<tr>
<td>HBV DNA PCR</td>
<td>10</td>
</tr>
<tr>
<td>HBeAg</td>
<td>9</td>
</tr>
<tr>
<td>HBeAg (+) DNA PCR (-)</td>
<td>1</td>
</tr>
<tr>
<td>HBeAg (-) DNA PCR (+)</td>
<td>2</td>
</tr>
<tr>
<td>HCV RNA PCR &amp; HBsAg</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 21. Liver Biopsy & Needle Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of biopsy (cm)</td>
<td>1.15 ± 0.64, Median 1.00</td>
<td></td>
</tr>
<tr>
<td>No of fragments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single fragment</td>
<td>44</td>
<td>(50%)</td>
</tr>
<tr>
<td>Two fragments</td>
<td>20</td>
<td>(22.7%)</td>
</tr>
<tr>
<td>More than two fragments</td>
<td>24</td>
<td>(27.3%)</td>
</tr>
<tr>
<td>Needle Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surecut Needle (Modified Menghini)</td>
<td>79</td>
<td>(88.8%)</td>
</tr>
<tr>
<td>Trucut Needle</td>
<td>1</td>
<td>(1.1%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>8</td>
<td>(9.1%)</td>
</tr>
<tr>
<td>Needle Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surecut 18 Gauge</td>
<td>76</td>
<td>(86.4%)</td>
</tr>
<tr>
<td>Surecut 16 Gauge</td>
<td>3</td>
<td>(3.4%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>9</td>
<td>(10.2%)</td>
</tr>
<tr>
<td>Scoring System</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knodell HAI</td>
<td>82</td>
<td>(93%)</td>
</tr>
<tr>
<td>Modified Knodell (Ishak)</td>
<td>6</td>
<td>(7%)</td>
</tr>
<tr>
<td>Category</td>
<td>Count</td>
<td>Percentage</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Stage of Fibrosis, Knodell HAI (Metavir)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 0 (F0) No Fibrosis</td>
<td>26</td>
<td>29.5%</td>
</tr>
<tr>
<td>Stage 1 (F1) Portal Fibrosis</td>
<td>39</td>
<td>44.3%</td>
</tr>
<tr>
<td>Stage 2 (Ishak) (F2) Portal fibrosis + septa</td>
<td>3</td>
<td>3.4%</td>
</tr>
<tr>
<td>Stage 3 (F3) Bridging Fibrosis</td>
<td>18</td>
<td>20.5%</td>
</tr>
<tr>
<td>Stage 4 (F4) Cirrhosis</td>
<td>2</td>
<td>2.3%</td>
</tr>
<tr>
<td><strong>Activity (Knodell Grade)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal activity (0-3)</td>
<td>11</td>
<td>12.5%</td>
</tr>
<tr>
<td>Mild activity (3-5)</td>
<td>33</td>
<td>37.5%</td>
</tr>
<tr>
<td>Moderate activity (6-11)</td>
<td>39</td>
<td>44.3%</td>
</tr>
<tr>
<td>Severe activity (&gt; 11)</td>
<td>5</td>
<td>5.7%</td>
</tr>
<tr>
<td><strong>Category of Fibrosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significant Fibrosis (F2-F4)</td>
<td>23</td>
<td>26.1%</td>
</tr>
<tr>
<td>Advanced Fibrosis (F3-F4)</td>
<td>20</td>
<td>22.72%</td>
</tr>
<tr>
<td>Insignificant Fibrosis (F0-F1)</td>
<td>65</td>
<td>73.9%</td>
</tr>
<tr>
<td><strong>Category of Activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significant Activity (Moderate-Severe)</td>
<td>44</td>
<td>50%</td>
</tr>
<tr>
<td>Steatosis</td>
<td>42</td>
<td>47.7%</td>
</tr>
</tbody>
</table>
**Table 23. Viral Aetiology and Histopathology**

<table>
<thead>
<tr>
<th></th>
<th>HCV</th>
<th>HBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Significant Fibrosis (F3-F4)</td>
<td>18/75 (24%)</td>
<td>6/14 (43%)</td>
</tr>
<tr>
<td>Significant Activity (Mod-Severe)</td>
<td>36/75 (48%)</td>
<td>9/14 (64%)</td>
</tr>
<tr>
<td>Steatosis</td>
<td>39/75 (52%)</td>
<td>3/14 (21%)</td>
</tr>
</tbody>
</table>

**Table 24. Lipid profile and HCV**

<table>
<thead>
<tr>
<th>Anti HCV</th>
<th>Negative</th>
<th>Positive</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.46 ±.39</td>
<td>4.56 ±.45</td>
<td>0.43</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>2.03 ±.80</td>
<td>1.67 ±.29</td>
<td>.004</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.01 ±.19</td>
<td>0.94 ±.07</td>
<td>.21</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.70 ±.50</td>
<td>2.92 ±.39</td>
<td>.79</td>
</tr>
</tbody>
</table>
Table 25. Clinical/Ultrasonographic/Biopsy Parameters vs Significant Fibrosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stage F0-F1</th>
<th>Stage F2-F4</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>31.2 ± 5.46</td>
<td>35.9 ± 6.88</td>
<td>0.001</td>
</tr>
<tr>
<td>Gender, Female, %</td>
<td>32 (49.2%)</td>
<td>9 (39.1%)</td>
<td>0.404</td>
</tr>
<tr>
<td>Ultrasound variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver echogenicity (increased)</td>
<td>12 (18.5%)</td>
<td>10 (43.5%)</td>
<td>0.017</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>5 (7.7%)</td>
<td>8 (34.8%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Portal vein diameter</td>
<td>2 (3.1%)</td>
<td>1 (4.3%)</td>
<td>0.773</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>2 (3.1%)</td>
<td>3 (13%)</td>
<td>0.076</td>
</tr>
<tr>
<td>Biopsy variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No to Mild Activity (necroinflammatory)</td>
<td>42 (64.6%)</td>
<td>2 (8.7%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Mod to Severe Activity</td>
<td>23 (34.5%)</td>
<td>21 (91.3%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Steatosis</td>
<td>27 (41.5%)</td>
<td>15 (65.2%)</td>
<td>0.051</td>
</tr>
</tbody>
</table>
### Table 26. Biomarkers vs Fibrosis categories (statistically significant)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stage F0-F1</th>
<th>Stage F2-F4</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>11.2 ± 4.55</td>
<td>13.7 ± 4.71</td>
<td>0.026</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>40.4 ± 12.68</td>
<td>51.6 ± 24.89</td>
<td>0.49</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>33.7 ± 12.1</td>
<td>53.4 ± 22.89</td>
<td>0.001</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>14.3 ± 0.73</td>
<td>15.4 ± 0.99</td>
<td>0.000</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>0.94 ± 0.06</td>
<td>1.00 ± 0.15</td>
<td>0.017</td>
</tr>
<tr>
<td>IgG (g/L)</td>
<td>13.8 ± 2.61</td>
<td>15.1 ± 2.48</td>
<td>0.040</td>
</tr>
<tr>
<td>Hyaluronic Acid (µg/L)</td>
<td>31.2 ± 16.63</td>
<td>73.9 ± 32.73</td>
<td>0.000</td>
</tr>
<tr>
<td>Alpha 2 Macroglobulin (g/L)</td>
<td>2.3 ± 2.17</td>
<td>3.9 ± 2.36</td>
<td>0.004</td>
</tr>
<tr>
<td>Platelets (x 10^9/L)</td>
<td>267 ± 45.98</td>
<td>199 ± 45.88</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Table 27. Biomarkers vs Fibrosis Categories (other)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stage F0-F1</th>
<th>Stage F2-F4</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>88.2 ± 37.22</td>
<td>93.6 ± 50.60</td>
<td>0.58</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>43.3 ± 5.45</td>
<td>43.2 ± 4.08</td>
<td>0.95</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>4.12 ± 0.73</td>
<td>4.14 ± 0.95</td>
<td>0.89</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.54 ± 0.45</td>
<td>4.58 ± 0.43</td>
<td>0.68</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.6 ± 0.29</td>
<td>1.8 ± 0.66</td>
<td>0.049</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.8 ± 0.42</td>
<td>2.9 ± 0.37</td>
<td>0.15</td>
</tr>
<tr>
<td>Globulins (g/L)</td>
<td>22.5 ± 7.46</td>
<td>23.1 ± 5.02</td>
<td>0.69</td>
</tr>
</tbody>
</table>
Table 28. Platelet Parameters vs Advanced Fibrosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stage F0-F2</th>
<th>Stage F3-F4</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count (x10⁹/L)</td>
<td>266 ± 45.47</td>
<td>193 ± 46.87</td>
<td>0.000</td>
</tr>
<tr>
<td>MPV (f/L)</td>
<td>10.6 ± 1.35</td>
<td>11.2 ± 1.08</td>
<td>0.076</td>
</tr>
<tr>
<td>PDW (f/L)</td>
<td>12.3 ± 1.62</td>
<td>13.6 ± 1.96</td>
<td>0.004</td>
</tr>
<tr>
<td>PDW Index</td>
<td>5.7 ± 1.53</td>
<td>9.5 ± 3.67</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Table 29. Logistic Regression Analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>Wald Test</th>
<th>p value</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>0.189</td>
<td>3.824</td>
<td>.051</td>
<td>1.208</td>
<td>1.000-1.459</td>
</tr>
<tr>
<td>GGT</td>
<td>0.120</td>
<td>5.768</td>
<td>.016</td>
<td>1.128</td>
<td>1.022-1.244</td>
</tr>
<tr>
<td>Hyaluronic Acid</td>
<td>0.080</td>
<td>8.796</td>
<td>.003</td>
<td>1.084</td>
<td>1.028-1.143</td>
</tr>
<tr>
<td>Alpha 2 Macroglobulin</td>
<td>0.518</td>
<td>6.032</td>
<td>.014</td>
<td>1.678</td>
<td>1.110-2.537</td>
</tr>
<tr>
<td>Platelets</td>
<td>-.040</td>
<td>5.694</td>
<td>.017</td>
<td>.960</td>
<td>.929-.993</td>
</tr>
<tr>
<td>Constant</td>
<td>-4.795</td>
<td>1.289</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Graph. 1 Receiver Operating Characteristic (ROC) curve Fibroscore vs F3 Fibrosis

Area under the ROC .938 (95% CI; .888-.988)
Graph. 2 ROC curve, Fibroscore vs F2 Fibrosis
Area under the ROC .808 (95% CI; .613-1.00)
Graph 3. Boxplot of predicted probability of Fibrosis (Fibroscore) vs various Stages of Fibrosis.

The Fibroscore range is from 0.00-1.00

The line through the box is the Median; the top and bottom edges of each box represent 25\textsuperscript{th} and 75\textsuperscript{th} percentile, giving the interquartile range; the vertical lines on either side of box represent distribution from the quartile to the farthest observation.
Graph 4. Boxplot of Hyaluronic Acid values vs different fibrosis stages.
Graph 5. Boxplot of Alpha 2 Macroglobulin values vs different Fibrosis stages
Graph 6. ROC curve of PDW Index vs F3 Fibrosis
Area under the ROC .801 (95% CI; .671-.930)
Graph 7. APRI and PDW Index ROC vs Stage F3-F4 Fibrosis

APRI area under the ROC .888 (95% CI; .797-.980)
PDW Index area under the ROC .840 (95% CI; .721-.958)
Graph 8. Boxplot of Platelets vs various Stages of Fibrosis
Graph 9. Boxplot PDW Index vs various stages of Fibrosis
SUMMARY

&

CONCLUSION
SUMMARY

1. With the increasing number of patients suffering from chronic liver disease from HBV and HCV, which may progress to cirrhosis with all its complications; clinicians and patients require accurate information about the degree of liver fibrosis, to guide management decisions, monitor disease activity, and predict outcome.

2. Many treatment algorithms recommend treatment for patients with septal fibrosis or greater (≥ stage F2, Metavir/Ishak), this recommendation also depends on viral cause, and particular viral subtype. However more efficacious treatments or improvements in tolerability of therapy could easily alter this treatment paradigm; increasing the physician and patient acceptability of therapy, and reducing the need for liver biopsy.

3. There is no ideal reference for the assessment of liver histology.

4. Liver biopsy, which is considered the ultimate standard, has three major limitations: a risk of adverse events, sampling error, and intra and inter observer variability. Liver biopsy is only 80% accurate in assessing stage of liver fibrosis, and could miss advanced fibrosis and cirrhosis in as high as 30% of patients. The factors associated with this inaccuracy of liver biopsy include: the heterogeneous nature of chronic liver disease; the relatively small size of liver biopsy sample compared to the size of liver (1/50,000), or even the size of a nodule as in macronodular cirrhosis, which is ≥ 3mm; and the experience of the histopathologist.

5. These limitations of biopsy have led clinical investigators to study alternative methods to investigate liver disease.

6. It is vital that new methods for the assessment of liver fibrosis be developed and validated, so that the liver fibrosis is diagnosed and impact of any new therapies can be rapidly assessed in a simple and meaningful way.

7. Noninvasive markers are the most widely used alternative to liver biopsy to stage chronic liver disease.
8. Serum markers of liver fibrosis offer an attractive alternative. They are less invasive than biopsy, with no risk of complications, eliminate sampling and observer variability, may allow dynamic calibration of fibrosis (liver biopsy is just a snap shot in time), and can be performed repeatedly.

9. There are two kinds of noninvasive markers: direct and indirect. There are advocates of both types of tests, but they are complementary to each other.

10. The ideal features of these serum markers are: noninvasive, liver specific, correlates well with the severity of liver disease especially fibrosis, reflective of fibrosis irrespective of cause, not confounded by other co-morbidities, minimally influenced by urinary and biliary excretion, easy to perform, and be commonly used in all patients.

11. There are three commercially available serum marker panels, which have been most extensively validated (in thousands of patients); Fibrotest, FibroSpect, and the European Liver Fibrosis Study Group Assay. Additionally, there have been many studies of noninvasive markers of liver fibrosis.

12. These tests are extremely accurate > 95% in determining the near absence (F0-F1) of fibrosis in CLD, and the presence of cirrhosis. They are also very accurate for advanced fibrosis (F3-F4); the predictability is more than 85%-90%, and the area under the ROC more than 0.90; but not for intermediate stages. Overall, the majority of noninvasive marker panels have an area under the ROC of 0.80-0.85 not for staging the disease, but differentiating mild (F0-F1) from clinically significant (F2-F3-F4) fibrosis; which is a significant advancement.

13. The relatively lower diagnostic performance in intermediate stages is related to liver biopsy itself. Since the noninvasive marker is validated against the biopsy, and the overall accuracy of biopsy is only 80%, it is probably statistically impossible for a marker to perform any better than biopsy.

14. This is not surprising, since these stages are relatively artificial separations of a spectrum of a disease process with progression in severity expressed as a categorical variable from 0-4 to 6. This does not accurately reflect the dynamic biologic process of fibrosis, and constrains the serum marker test performances that are capable of generating continuous variables.
15. The noninvasive markers also correlate fairly well with the necroinflammatory activity.

16. In addition to diagnosing hepatic fibrosis, these tests have proved valuable in predicting changes in fibrosis content over time in individual patients. Those patients who favorably respond to therapy have changes in mean serum values of fibrosis markers. Thus, these assays may be useful not only in the initial staging of the liver disease, but may also be of value in following the histological response to therapy.

17. The long term (5 year) prognostic value of one of the test panels (Fibrotest) is at least equal to that of liver biopsy.

18. Thus with the use of serum markers: the severity of fibrosis is diagnosed; the stage of fibrosis helps predict the likelihood of response to therapy in CLD from HBV and HCV, since advanced stages of fibrosis generally have a lower response; treatment may be delayed or deferred if little fibrosis progression has occurred over time, in certain HCV genotypes such as genotype 1; and approximate time to the development of cirrhosis can be estimated.

19. Besides, the noninvasive markers allow a quantitative estimation of fibrosis, which is a major breakthrough.

20. Although remarkable progress has been achieved in the technique of noninvasive markers, there are some caveats.

21. These tests must be interpreted in the overall clinical and biological context. The serum markers, especially the direct markers typically reflect the rate of matrix turnover, not deposition and thus tend to be more elevated when there is high inflammatory activity. None of the markers are liver specific, so their serum levels may be affected by concurrent inflammation, fibrosis elsewhere, or unrelated phenomenon such as haemolysis. Their serum levels may be affected by clearance rates, such as in impaired renal or biliary excretion. Further refinements in technique and even better noninvasive marker algorithms will decrease the risks of false positives and false negatives.

22. An alternative method to stage chronic liver disease is elastography, or measurement of hepatic elasticity or stiffness.
23. The sensitivity of noninvasive markers is higher for early stage fibrosis, but elastography is helpful because of its specificity for severe fibrosis.

24. This technique, however, has its limitations: it uses expensive equipment, and has decreased accuracy in obese patients and those who have ascites.

25. Nevertheless, elastography is complementary, as the combination of noninvasive markers and elastography improves the overall accuracy.

26. The development of noninvasive markers derived from proteomics and genomics is likely to be important in the years to come.

27. Several published studies and overviews have demonstrated the predictive value and better benefit-to-risk ratio than liver biopsy of these combinations of simple serum noninvasive markers in patients with chronic liver disease. They allow a quantitative assessment of fibrosis according to the cause of liver disease, as well as an assessment of the necroinflammatory activity. These tests which are now available worldwide, can facilitate not only the screening and management of fibrotic liver disease, but also provide useful prognostic value, and will be extremely helpful to the clinicians.

28. It is probable that in not too distant future, the combination of noninvasive marker panels and/or noninvasive markers + elastography could replace liver biopsy in most patients with chronic hepatitis.
CONCLUSION

Chronic viral hepatitis is a common disease in the general population of which the world is facing a pandemic. In the last decade, advances in serological and virological testing, and improvements in therapy have led more patients to be identified and to seek treatment. In chronic viral hepatitis the prognosis is highly dependent on the extent and progression of hepatic fibrosis. Thus accurate information about the degree of fibrosis is required to guide management decisions, monitor disease activity, and predict outcome.

The clinicians rely on liver biopsy for both prognostic and therapeutic decision making, which can have a major impact on patient’s life. Though classically considered the “gold standard”; the liver biopsy is far from perfect, and has significant limitations. This has led researchers to look for other methods to assess the stage of liver fibrosis. The noninvasive markers are the most widely used alternative to liver biopsy; and tremendous progress has been made in this regard.

In the study presented, the Fibroscore results are extremely accurate (> 95%) in determining the near absence of fibrosis in viral related chronic liver disease, and cirrhosis. Fibroscore also correlates highly with clinically significant fibrosis, and provides useful assessment of the necroinflammatory activity. The association between platelet parameters and fibrosis was also looked into; the PDW Index reliably predicts advanced fibrosis.

Thus the Fibroscore results have high diagnostic value to stage fibrosis; for either the presence or exclusion of very early stage, clinically significant, or advanced stages of fibrosis. These tests will assist in the screening and management of fibrotic liver disease; and represent a valid alternative to liver biopsy.

It is concluded that, the noninvasive markers will replace liver biopsy in most patients with chronic liver disease from hepatitis B and C viruses.