ACCURACY OF LIVER STIFFNESS MEASUREMENT USING FIBROSCAN ® TO PREDICT THE RESPONSE TO ANTIVIRAL THERAPY IN PATIENTS WITH CHRONIC HEPATITIS C VIRAL INFECTION

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1. Hepatitis C Virus

1.1 HCV structure and lifecycle

In the 1989, Hepatitis C virus (HCV) was initially isolated from the serum of a person with non-A, non-B hepatitis by Choo et al (1). HCV is a small (55–65 nm in size), enveloped, positive-sense single-stranded RNA virus of the family Flaviviridae. It consists of a core of genetic material (RNA), surrounded by an icosahedral protective shell of protein, and further encased in a lipid (fatty) envelope of cellular origin (2). Two viral envelope glycoproteins, E1 and E2, are embedded in the lipid envelope. The HCV genome (Figure 1) consists of a positive-stranded RNA molecule of about 9.6 kilobases and encodes a large polyprotein precursor (about 3000 amino acids). This precursor protein is cleaved by the host and viral protease to generate at least 10 proteins: the core, envelope 1 (E1), E2, p7, nonstructural (NS) 2, NS3, NS4A, NS4B, NS5A, and NS5B that allow viral replication within the host cell, or assemble into the mature viral particles (3).

Figure 1: HCV genome and the specific cleavage proteins.
Translation of HCV proteins requires an internal ribosome entry site (IRES) located in the 5′ untranslated region of the viral mRNA. The core domain of HCV IRES contains a four-way helical junction that is integrated within a predicted pseudoknot (4). The conformation of this core domain constrains the open reading frame's orientation for positioning on the 40S ribosomal subunit (5).

HCV nonstructural proteins and viral RNA have been detected in livers of infected patients or experimentally inoculated chimpanzees, confirming that the liver is a site of HCV replication (6). The virus replicates mainly in the hepatocytes of the liver, where it is estimated that daily each infected cell produces approximately fifty virions (virus particles) with a calculated total of one trillion virions generated. The virus may also replicate in peripheral blood mononuclear cells both in vivo and ex vivo or in experimentally infected B- and T-cell lines, potentially accounting for the high levels of immunological disorders found in chronically infected HCV patients (7).

The genome of HCV is highly mutable. Because HCV is an RNA virus and lacks efficient proofreading ability as it replicates, virions infecting humans undergo evolution with time, giving rise to the notion that HCV persists as a collection of virus quasispecies. By constant mutation, HCV may be able to escape host immunologic detection and elimination (8). HCV undergoes rapid mutation in a hypervariable region of the genome coding for the envelope proteins and escapes immune surveillance by the host so that they are no-longer recognized by T cells and neutralizing antibodies, in addition to interfering with host cell cellular components and signaling pathways (9). Consequently, most HCV-infected people develop chronic infection. Mutations are not randomly distributed along the genome, but are most pronounced within a hypervariable region located near the N-terminus of E2. This region maps at a surface loop of the E2 protein containing a B-cell epitope that undergoes antigenic evolution over time. The generation of escape variants is one of the most potent immune evasion strategies utilized by HCV (9).

Based on genetic differences between HCV isolates, the hepatitis C virus species is classified into seven genotypes (1–7) with several subtypes within each genotype represented by lower-cased letters. Subtypes are further broken down into quasispecies based on their genetic diversity (10). Genotype 1 is the most prevalent
worldwide with a higher proportion of genotype 1b in Europe and 1a in the USA. Genotype 3 is more prevalent among injection drug users. Genotype 2 is found in clusters in the Mediterranean region, while 5 and 6 are rare in Europe (11). Notably, infection with one genotype does not confer immunity against others, and concurrent infection with two strains is possible. When this happens, one of the strains removes the other from the host in a short time (12). Determination of viral genotype is mandatory in management as it can predict the response to antiviral therapy especially in interferon-based regimens as well as the duration of treatment (13).

1.2 Epidemiology and natural history of the HCV infection

HCV infection constitutes a major health concern, as it affects approximately 184 million patients with a prevalence of 2.35% and an incidence of 3-4 million new infections per year worldwide (14-16). The transmission of HCV is mainly through exposure to infected blood. Risks for transmission include: blood transfusion before, high-risk sexual activity, solid organ transplantation from an infected donor before the introduction of screening of blood donations in 1990, occupational exposure, hemodialysis, household exposure, intravenous drug use, and rather rarely vertical transmission from infected mother (17).

The diagnosis of HCV infection is usually obtained by detection of anti-HCV antibodies. However, the anti-HCV reactivity by screening assays can indicate a past, acute or chronic hepatitis and despite the high specificity of the assays (> 99%) false positive results are not rare. At this point, molecular determination of HCV-RNA is mandatory for the diagnosis of an active HCV infection (18).

Acute hepatitis C is rarely severe, and symptoms occur in 10 to 50% of cases. In Europe, HCV infection is responsible for about 10% of cases of acute hepatitis (19). The clinical outcomes after the initial HCV infection are highly variable. Most individuals are asymptomatic with chronic infection until the late stages of disease. Studies have demonstrated that about 20% of individuals infected with HCV will spontaneously clear the virus after initial infection. Host factors such as *IL28b* polymorphism genotype have been associated with spontaneous
clearance (20) as well as the female sex (21). Over many decades, (Chronic hepatitis C, CHC) can proceed towards cirrhosis. On average, 10 to 20% of patients develop cirrhosis over 20–30 years of HCV infection (22). The risk of developing HCC is approximately 1 to 5% per year. Patients diagnosed with HCC have a 33% probability of death during the first year after diagnosis (18, 23). Many studies have proved the association between fibrosis progression and some host factors such as: male gender, insulin resistance, immunosuppression and chronic alcohol consumption (24). Tobacco smoking has been found to increase inflammation and accelerates fibrosis (25). On the other hand, coffee consumption seems to have a positive effect as it is associated with lower inflammatory activity and even lower risk of development of (hepatocellular carcinoma, HCC) (26).

1.3 Complications of chronic hepatitis C virus infection

Cirrhosis is the end stage of chronic liver disease and it is irreversible. The development and consequences of cirrhosis depend on: 1) the complete disruption of the normal liver architecture with the formation of shunts and intrahepatic obstruction to the flow in the portal system, 2) progressive reduction of functional liver. Progressive loss of the liver functions and/or portal hypertension are, therefore, the basis of clinical manifestations and complications of the disease (27). The progression of cirrhosis is, thus, marked by the development of one or more complications. The main complications of liver cirrhosis are listed below.

Hepatic encephalopathy (28): hepatic or porto-systemic encephalopathy is a neuropsychiatric syndrome that is observed in patients with severely impaired hepatic function. The clinical spectrum is highly variable as it could range from subtle abnormalities detectable only by specific tests to profound changes in the level of consciousness or even coma. The pathogenic mechanisms are not fully known. The old hypothesis is that of the neurotoxic ammonia, alternative pathogenetic hypotheses indicates a possible role of altered equilibrium between the neurotransmitters of the central nervous system. Other theories suggest the possibility that changes in the concentration of short-chain fatty acids or altered permeability of the blood-brain barrier.

Ascites (29): it is the accumulation of fluid in the peritoneal cavity, is the most frequent complication of cirrhosis since it occurs in about 60% of patients within 10
years after disease diagnosis. The onset of ascites is considered as a negative prognostic significance. Its appearance indicates that there are in fact profound alterations in systemic and splanchnic hemodynamics and renal function that occur as a result of portal hypertension. The presence of ascites may also favor the occurrence of further complications such as electrolyte imbalances, renal failure (the so-called "hepatorenal syndrome") and spontaneous bacterial peritonitis (30).

Gastrointestinal bleeding (31): bleeding from the upper gastrointestinal tract is a frequent complication in patients with cirrhosis and it is the leading cause of death in 25-30% of cases. In the patient with liver cirrhosis who developed esophageal or gastric varices, the risk of bleeding is about 4% per year when the esophageal varices are small, but the risk is doubled (about 9% per year) if varices have a greater caliber. Finally, bleeding may be also a non-hypertensive one, which is not directly related to portal hypertension but due to concomitant diseases (peptic ulcer, hiatal hernia, Mallory-Weiss syndrome, hemorrhagic erosive gastritis or cancer) that may be more frequent in the cirrhotic.

Hepatorenal syndrome (32-34): it is well known that the kidneys can be affected in many different ways as a result of the (end stage liver disease, ESLD). IgA nephropathy, membranous glomerulonephritis and cryoglobulinemia are among the most common renal dysfunctions that might affect patients with ESLD. (Hepatorenal syndrome, HRS) comprises the most advanced stage of hemodynamic dysfunction that is characterized by renal vasoconstriction in the setting of splanchnic and systemic arterial vasodilatation. Clinically, there are two variants of HRS:
- Type 1 HRS is characterized by rapidly progressive renal failure, with a doubling of serum creatinine to a level greater than 2.5 mg/dL over a period of less than two weeks.
- Type 2 HRS is slower in onset and progression. It is defined by an increase in serum creatinine level to >1.5 mg/dL or a creatinine clearance of less than 40 mL/min, and a urine sodium < 10 μmol/L.
The prognosis of patients with HRS is grim; untreated patients have an extremely short survival.
Figure 2: schematic demonstrating the underfill theory to explain the pathophysiology of both ascites and hepatorenal syndrome.

Spontaneous bacterial peritonitis (35): spontaneous bacterial peritonitis is bacterial infection of the ascitic fluid which occurs without any apparent intra-abdominal source of infection. It can complicate ascites, whatever its etiology, but is particularly common in cirrhotic patients in which it has an incidence ranging from 8 to 27% of cases, while it is rare in cardiogenic ascites. Left untreated, the prognosis is rather grim. However, in recent years, thanks to advances in treatment, including intensive, but especially to the early diagnosis, hospital mortality decreased from 100% to 20-40%. The onset pf HRS is considered one of the predictors of hospital
mortality. In fact, approximately 50% of patients with spontaneous bacterial peritonitis that developed renal failure died during hospitalization compared with 6% of patients with normal renal function. However, the long-term prognosis of those who survive an episode of spontaneous bacterial peritonitis is poor. The main causes of death are the ESLD and recurrent bacterial peritonitis (40-70% of cases/year).

1.4 Treatment of CHC

The primary goal of HCV therapy is to cure the infection. A sustained virological response (SVR) is defined as undetectable HCV RNA 12 weeks (SVR12) or 24 weeks (SVR24) after treatment completion. The infection is cured in more than 99% of patients who achieve an SVR (36). The SVR is generally associated with resolution of liver disease in patients without cirrhosis. Patients with cirrhosis remain at risk of life-threatening complications; however hepatic fibrosis may regress and the risk of complications such as hepatic failure and portal hypertension is reduced (36).

The combination of pegylated interferon (IFN)-α and ribavirin for 24 or 48 weeks was considered the gold standard for the treatment of CHC (37). With this regimen, patients infected with HCV genotype 1 had SVR rates of approximately 40% - 50%. Higher SVR rates were attained in patients infected with HCV genotypes 2, 3, 5, and 6 (up to about 80%, and higher for genotype 2 than for genotypes 3, 5, and 6) and intermediate SVR rates were achieved in those with HCV genotype 4 (38). In addition to the patient population that is not cured by the available regimens, is the burden of numerous patients who go untreated due to contraindications (advanced hepatic disease, autoimmune disease, and psychiatric illness) or refusal to receive interferons, as well as poor compliance or discontinuation of therapy due to adverse effects (fatigue, headache, fever, cytopenia, autoimmune disorders, insomnia, and depression). Other downsides of interferons include their need to be injected and the long duration of treatment.

In 2011, FDA approved two new protease inhibitors for HCV, telaprevir and boceprevir, both used in combination with PEG-IFN and RBV, dramatically improve
efficacy and can cure up to 75% of patients chronically infected with Genotype-1 HCV. Although regimens containing protease inhibitors have resulted in higher SVR and shorter duration of treatment, their limitations include a low genetic barrier to resistance, more side effects, complex medication regimens, and a potential for drug-drug interactions.

Improved understanding of HCV replication and life cycle has allowed for the development of a plethora of new therapeutic agents that target enzymes directly, directly acting antivirals (DAAs). This life cycle has several important steps which can be targeted, and eventually interrupted, by DAAs including: the first generation NS3/4A serine protease inhibitors (e.g., TPV and BOC); the second generation NS3/4A protease inhibitors (e.g., simeprevir, faldaprevir and ABT-450); the NS5A replication complex inhibitors (e.g., ledipasvir, daclatasvir and ABT-267); the NS5B non-nucleoside polymerase inhibitors (e.g., sofosbuvir and ABT-333) and nucleoside inhibitors (e.g., Mericitabine). AP89652 is a recently discovered molecule identified by Dufner-Beattie et al. by screening a compound library with an HCV Gt1b subgenomic replicon assay. The viral protein target of this compound was identified as NS4B (39).

The current interferon-free regimens approved or under clinical trials have substantially higher SVR rates compared with standard PEG-IFN/RBV treatment even in genotype 1 infection with lower treatment duration, decreased pill burden and adverse events, no injection and higher SVR rates. However, it is a major limitation that DAAs are very expensive. Indeed, development of DAAs obviously led to challenge and change in the paradigm of management for hepatitis C patients.
Chapter II:

2. Liver Fibrosis

2.1 Liver fibrosis: definitions and mechanisms

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 (40, 41). Fibrosis is the deposition of extracellular matrix, ECM, components within the liver parenchyma which leads to distortion of the liver architecture with formation of fibrous scar tissue (40).

Any cause of liver injury, such as that seen in CHC, may lead to regeneration of parenchymal cells which is superseded by an increase in deposition of ECM proteins which eventually replaces the normal hepatocytes. The progression of fibrosis occurs in a stepwise fashion. In CHB, the fibrotic tissue initially surrounds the portal tracts. With more advance disease and with ongoing chronic injury, portal-portal bridging of fibrosis occurs which may eventually progress to cirrhosis.

Recently, notable progress has been made in the understanding of the pathophysiology of 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 (42, 43).

2.2 Cellular Sources of Extracellular Matrix – Normal and Fibrotic Liver

i) Hepatic stellate cells (HSCs) are the major source of ECM deposition, although other cell types such as portal fibroblasts may play a role. The HSC’s can be activated by several cytokines (e.g. tumor growth factor beta (TGF-b), tumor necrosis factor alpha (TNF-a), 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. 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-b1, PDGF, monocyte chemotactic protein 1 (MCP-1), and endothelin (ET-1). Some of these are directly involved in the fibrogenesis (TGF-b1, connective tissue growth factor), others in chemotaxis (MCP-1) and proliferation of HSC’s (PDGF, ET-1), and others in matrix degradation (metalloproteinases) (43). HCV 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.

ii) Sinusoidal Endothelial Cell: ECM 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 notable heterogeneity in normal and fibrotic liver. Endothelial cells from normal liver produce type III, and type IV collagens, laminin, syndecans, and fibronectin. After 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 HSCs (44, 45).

A complex interaction between different cell lines occurs during hepatic fibrogenesis. The damaged hepatocytes produce reactive oxygen species (ROS) and fibrogenic mediators with consequent recruitment of inflammatory cells. The apoptosis of damaged hepatocytes stimulates the production of collagen by the liver myofibroblasts (46). The inflammatory cells and lymphocytes activate the HSCs resulting in the production of collagen (47). Activated HSCs secrete inflammatory chemokines, express cell adhesion molecules and modulate lymphocyte activation (48). It is a vicious circle, the HSCs and inflammatory cells stimulate each other (49). The fibrogenic process is also influenced by the different subtypes of T-helper lymphocytes present, being the Th2 response associated with a more active fibrogenesis (50). Kupffer cells are resident macrophages also play an important role in liver inflammation by releasing ROS and cytokines (51). Lastly, changes in the composition of the ECM are able to directly stimulate fibrogenesis. The type IV collagen, fibrinogen and plasminogen activator type urokinase stimulate hepatic
stellate cells by activating latent cytokines such as TGF-1 (52). Collagen fibers bind and stimulate hepatic stellate cells through the receptor DDR2. The cytokines that regulate the inflammatory response to the damaging stimulus modulate hepatic fibrogenesis both in vivo and in vitro (53). The monocyte chemotactic protein type 1 stimulates fibrogenesis, while the IL10 and IFN exert the opposite effect (54). Among the growth factors, TGF-appears to be a key mediator in human fibrogenesis. The TGF-promotes the transformation of HSCs into myofibroblasts, stimulating the synthesis of ECM proteins and inhibits their degradation (55). The platelet-derived growth factor (PDGF) is the most potent mitogen for HSCs and its production has increased in fibrotic liver (56).

Cytokines with vasoactive properties regulate hepatic fibrogenesis. Vasodilatory substances (eg. Nitric oxide, relaxin) exert antifibrotic effects, while vasoconstrictor substances (eg. Norepinephrine, angiotensin II) have opposite effects (57). Among vasoactive cytokines angiotensin II appears to play an important role in hepatic fibrogenesis. Angiotensin II is the effector peptide of the renin-angiotensin system, which is the main system responsible for the control of blood pressure in humans. The essential components of this system are locally expressed in chronically damaged liver and activated HSCs which produce angiotensin II (58). Angiotensin II causes liver inflammation and stimulates the proliferation and migration of HSCs, and the production of pro-inflammatory cytokines and collagen synthesis (58). Adipokines, cytokines produced by adipose tissue, regulate hepatic fibrogenesis. Leptin is essential for the activation of hepatic stellate cells and the development of fibrosis (53).

As mentioned previously, the fibrosis is a response to liver damage by any cause. As a result of the damaging stimulus, the inflammatory cells infiltrate the hepatic parenchyma, hepatocytes undergo apoptosis and Kupffer cells are in turn activated releasing fibrogenic mediators. HSCs are activated and proliferate, secreting a large amount of ECM proteins. Sinusoidal endothelial cells lose their fenestrations and tonic contraction of HSCs causes increase in resistance to blood flow at the level of liver sinusoids.
2.3 Assessment of the degree of liver fibrosis

2.3.1 Liver biopsy

Liver biopsy has been considered the "gold standard" for the diagnosis, staging and follow-up of liver diseases and is currently the standard of comparison between different diagnostic noninvasive markers of fibrosis. There are two different techniques for obtaining samples of liver tissue, i.e. the wedge biopsy and needle biopsy. The percutaneous needle biopsy technique is faster, safer and more used for the study, grading and staging of diffuse liver disease. In addition, most of clinicians today use the ultrasound-assisted percutaneous biopsy or ultrasound-guided to the significant reduction of the complications. This procedure is especially useful to define the etiology of the liver disease, determine the proper management and to exclude alternative diagnoses or comorbidities (60).

2.3.2 Histological grading and staging

Histological grading and staging are widely used in the assessment of liver biopsies from patients with chronic viral hepatitis, particularly hepatitis C.

The grading is a histological evaluation of hepatic necroinflammation. The following histological features must be assessed in order to define it (61):
1. The nature of the inflammatory infiltrate (consisting of lymphocytes and rare plasma cells) at the level of the portal tracts, including the branches of the hepatic artery and portal vein and the interlobular bile ducts.
2. The nature of periportal necrosis and destruction of the lamina limiting periportal hepatocytes induced by inflammatory cells, the characteristic of interface hepatitis or "piecemel necrosis".
3. The nature of confluent necrosis that exceeds the limiting lamina and joins or forms bridges between vascular structures, ie spaces between portals and portal spaces or, more importantly, between portal tracts and the center-lobular vein, with the appearance of the characteristic necrosis bridge or "bridging necrosis".
4. The nature of degeneration of hepatocytes and focal necrosis within the lobule.
The staging is a histological evaluation of the extent of liver fibrosis which reflects the level of disease progression. Several histologic classification systems have been proposed to minimize these uncertainties and 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 (62). The most common quantitative system that has been used in the assessment of chronic viral hepatitis is Knodell score (10), figure 3.
Knodell score (histology activity index score) of liver biopsy specimens for necrosis, inflammation, and fibrosis*

<table>
<thead>
<tr>
<th>I. Periportal bridging necrosis</th>
<th>II. Intralobular degeneration and focal necrosis*</th>
<th>III. Portal inflammation</th>
<th>IV. Fibrosis</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild piecemeal necrosis</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Moderate piecemeal necrosis</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(involves less than 50 percent of the circumference of most portal tracts)</td>
<td></td>
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<tr>
<td>Marked piecemeal necrosis</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
<td>(involves more than 50 percent of the circumference of most portal tracts)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Moderate piecemeal necrosis</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus bridging necrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marked piecemeal necrosis</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus bridging necrosis</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Multilobular necrosis§</td>
<td>10</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* The histology activity index score is the combined scores for necrosis, inflammation, and fibrosis.

* Degeneration-acidophilic bodies, balloononing; focal necrosis-scattered foci of hepatocellular necrosis.

* Loss of normal hepatic lobular architecture with fibrous septae separating and surrounding nodules.

* Bridging is defined as ≥2 bridges in the liver biopsy specimen; no distinction is made between portal-portal and portal-central linkage.

§ Two or more contiguous lobules with panlobular necrosis.

Another semiquantitative system, the METAVIR score is also used routinely in studies of chronic hepatitis C.

In METAVIR 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).

2.3.3 Noninvasive assessment of liver fibrosis

Noninvasive tests of hepatic fibrosis are primarily used for staging of fibrosis in patients with chronic liver disease. We typically consider noninvasive testing for patients with chronic viral hepatitis at the time of initial evaluation to determine the likelihood of advanced liver fibrosis. In patients who are not successfully treated, subsequent testing is useful to determine if there is progression of fibrosis. The tests are also being used in patients with other chronic liver diseases, such as nonalcoholic fatty liver disease and primary sclerosing cholangitis.

In patients with CHC, the assessment of fibrosis progression can be valuable for several reasons:

- The stage of fibrosis helps predict the likelihood of response to interferon-based therapy, since advanced stages of fibrosis (F3 or F4) generally have a lower response.
- Patients with cirrhosis require screening for hepatocellular carcinoma.
- To determine the necessity to undergo a liver biopsy.

2.3.2a Serologic tests:

A variety of serologic markers have been evaluated to predict the degree of fibrosis in the liver, and panels have been developed that combine assays of multiple
markers to improve predictive ability. The most studied panels are the aspartate aminotransferase (AST) to platelet ratio (APRI), FibroTest/FibroSure, Hepascore, and FibroSpect. Overall, studies of the various panels suggest that they have good ability to differentiate patients with significant fibrosis (F2 to F4) from those without significant fibrosis (F0 to F1) (65). A disadvantage of these panels is that they are not able to reliably differentiate between the different stages of fibrosis, and indeterminate outcomes are common (up to 50 % with the FibroTest). No panel has yet emerged as standard of care, and the choice of panel is often dictated by local availability.

2.3.2b Radiological tests:

Radiologic methods for staging hepatic fibrosis are emerging as promising tools. The methods include ultrasound-based transient elastography, magnetic resonance elastography, acoustic radiation force impulse imaging, and cross-sectional imaging. Ultrasound-based TE is the most studied radiologic method for staging hepatic fibrosis.

2.3.2c Transient elastography (ShearWave Elastography and FibroScan®)

Transient elastography (TE) is performed using transducer-induced vibrations at a low frequency (50 Hz) and amplitudes. The transmitted shear waves propagate through the liver parenchyma. Pulse-echo ultrasound acquisition is used to follow the propagation of the shear wave and to measure its average speed (66). Results are expressed in kPa and can range from 2.5 to 75 kPa (67). Cutoff values for diagnosing significant fibrosis (F≥2) or cirrhosis (F4) vary depending on the underlying liver disease. However, commonly used cutoffs in clinical settings are >7 kPa for significant fibrosis (F2 to F4) and >11 to 14 kPa for cirrhosis. TE does not allow differentiation between the contiguous stages of liver fibrosis (68).

Measurements are taken from the right lobe of the liver via the 9th, 10th, or 11th intercostal space (69). To minimize errors, TE should be performed from several sites (70). Measurements are taken from representative cylindrical areas
approximately 10 mm wide and 40 mm long. There are different sizes of probes, a standard M probe and an XL probe [for obese patients (71)].

TE has good inter- and intraobserver agreement in non-obese patients (69). However, interobserver agreement was lower in patients with mild fibrosis, steatosis, or an increased body mass index [BMI >25 kg/m² (69)]. Although liver biopsy remains the gold standard to assess liver fibrosis, TE is a good non-invasive alternative for determining the presence or absence of cirrhosis.

The Fibroscan® (Echosens, Paris, France) is a hardware used for liver stiffness measurement (LSM) by TE, and is the hardware used for LSM in this thesis. The major components of the Fibroscan include the probe which contains both the vibrating piston (typically 50 Hz) and the ultrasound transducer (5 MHz) mounted along the axis of the piston (figure 4). The probe is connected to a main computer and control unit (figure 5) which calculates the liver stiffness values and displays the results on a monitor. The displacement that is induced by this low-frequency wave is measured using standard cross-correlation technique. The axial displacement can then be estimated in each segment by comparison between successive radiofrequency lines. The region of interest used to calculate the velocity ranges from 2.5 to 4.5 cm below the skin surface, thereby avoiding subcutaneous adipose tissue and the liver fibrous capsule in the majority of cases.
Figure 4: The ultrasound transducer of the fibroscan
Figure 5: The fibroscan machine

TE was performed by placing an ultrasound transducer probe of FibroScan® on the intercostal space at the area of the right liver lobe with patients lying in a supine position with maximal abduction of the right arm. When the target area had been located, the operator pressed the probe button to commence the measurements.
The measurement depth is between 25 and 65 mm. Ten validated measurements should be performed on each patient.

2.3.3a Advantages of liver stiffness measurement

There are numerous major advantages of transient elastography compared with liver biopsy.

1. LSM is a non-invasive test and does not pose any risk of radiation to the patient. Therefore there is no known morbidity or mortality associated with TE.
2. The results are obtained immediately without the need to process specimen samples and the waiting time for the results to be interpreted by a histopathologist.
3. The patient does not require hospital admission.
4. LSM is obtained using 1/500th volume of the total liver, compared to 1/50,000th that is examined by liver biopsy (60). Therefore, a much larger area of the liver is surveyed, making sampling error less likely.

2.3.3b Limitations of liver stiffness measurement

1. TE is useful only for the evaluation of liver fibrosis by measuring LS, and in contrast to liver biopsy, it provides no diagnostic information about the underlying etiology of liver disease.
2. It does not provide information as to the degree of activity or inflammation that is present in the liver. On the other hand, liver biopsy can provide information on the activity of the underlying liver disease, such as the grade of hepatitis observed in many liver diseases, including CHC.
3. It is not advised for patients with ascites, and/or morbid obesity.

The pathophysiological mechanisms by HCV induced liver fibrosis are not well defined. Presumably, HCV induces oxidative stress and recall inflammatory cells that promote the activation of HSC and the accumulation of collagen. In addition, some
proteins of HCV directly stimulate the fibrogenic and inflammatory processes mediated by HSCs (59) bateller

Chapter 3

**Accuracy of liver stiffness measurement using FibroScan ® to predict the response to antiviral therapy in patients with chronic hepatitis C viral infection (HCV)**

3.1 Introduction:

Chronic hepatitis C (CHC) is generally characterized by a slow and long natural history with increasing degrees of fibrosis, culminating in liver cirrhosis. Emerging data demonstrate that antiviral therapy, particularly among those achieving a sustained virologic response (SVR), is associated with improved fibrosis and reduced incidence of cirrhosis and hepatocellular carcinoma. In the last decade, the combination of pegylated Interferon alfa-2 plus ribavirin was considered the standard-of-care treatment for CHC, irrespective of genotype. However, the rates of sustained virologic response (SVR) have not exceeded 45% in patients infected with HCV genotype 1 and up to 80% of patients with HCV Genotype 2 and 3. In 2011, the FDA approved two new inhibitors of non-structural 3/4A (NS3/4A) viral protease [Telaprevir (TPV) and Boceprevir (BOC)] for HCV Genotype 1. These two NS3/4A protease inhibitors associated with pegylated interferon and ribavirin have increased the rate of SVR up to 67% for BOC [72] and to 75% for TPV [73] in treatment-naïve patients. There are many factors that influence the response to antiviral treatment, including genotype, viral load and, the stage of fibrosis at the time of treatment. The gold standard for staging the hepatic fibrosis is liver biopsy. Although liver biopsy is generally safe, it is a costly procedure that carries a small risk of severe complications (diaphragm puncture, lung perforation, renal injury and hepatic artery branch injury with bleeding and/or hemoperitoneum) and it is also difficult to accept for patients. In addition, sampling error is common because only 1/50,000 of the
organ is analyzed, resulting in up to 30% of false negative results. These limitations may lead to an underestimation of cirrhosis, especially when liver biopsy specimens are small or fragmented. Among the non-invasive tests proposed for staging of liver fibrosis, the measurement of liver stiffness (LS) using transient elastography has been demonstrated to be a reliable tool.

FibroScan (Fibroscan®, Echosens, Paris, France) is a non-invasive device that assesses the stiffness of the liver via the technique of transient elastography (TE). Briefly, an ultrasound transducer probe is mounted on the axis of a vibrator. Vibrations of mild amplitude and low frequency (50 Hz) are transmitted by the transducer, inducing a plastic shear wave that propagates through the underlying tissues. Pulse-echo ultrasound acquisition is used to follow the propagation of the shear wave and to measure its velocity, which is directly related to tissue stiffness (the elastic modulus E expressed as \( E = 3\rho V^2 \), where \( V \) is the shear velocity and \( \rho \) is the mass density, which is constant for tissues). Simply, the stiffer the tissue, the faster the shear wave propagates (Figure 6).

**Figure 6:** Fibrosis grades and their corresponding stiffness measured in KPa

Once the measurement area has been located, the operator presses the button on the probe to start an acquisition. The software determines whether each measurement is valid or not. Results are expressed in kilopascals (kPa) and correspond to the median of 10 validated measurements according to Sandrin et al.
(74). TE measures liver stiffness in a volume that approximates a cylinder 1 cm in diameter and 4 cm in length, between 25 and 65 mm underneath the skin surface. This volume is at least 100 times bigger than a biopsy sample and therefore should be more representative of the liver parenchyma (75, 76). Therefore, it is considered to be a technique with a very high diagnostic accuracy and reproducibility to estimate liver fibrosis by LS measurement. TE has the advantages of being painless, rapid, more cost-effective than liver biopsy and easy to perform at the outpatient clinic.

Albeit accurate and easy to perform, TE confines its own limitations. Unfortunately, there are several factors that could contribute to LS other than fibrosis. Indeed, LS has been consistently found to be falsely elevated in acute hepatitis, manifested as alanine aminotransferase (ALT) flares (77). At this point, the results of TE can be misleading and TE is not recommended until at least 3 months after ALT normalization. Extrahepatic cholestasis (78), hepatic congestion (79), hepatic amyloidosis (80) and food intake (within 60 minutes) (81) were also found to be associated with a falsely high LS values. Apparently the degree of hepatic steatosis does not affect the LS, so it can be considered as an accurate surrogate in CHC patients with associated non-alcoholic fatty liver disease according to Arena et al and Wong et al (82, 83).

The aim of this prospective, non-randomized, uncontrolled cohort study was to assess the changes of liver stiffness measurement by Fibroscan® during the antiviral therapy for patients with CHC and whether the changes in liver stiffness can predict response to antiviral therapy.

3.2 Patients and Methods:

The study was carried out between January 2012 and November 2014. Consecutive CHC patients close to start antiviral therapy were enrolled before the beginning of treatment course. Their demographic and clinical characteristics as well as treatment regimens, duration and posology were obtained upon enrollment.

3.2.1 Inclusion criteria
• Age > 18 years;
• BMI <30 kg/m2;
• Diagnosis of chronic hepatitis C or HCV-related liver cirrhosis in class A of Child-Pugh
• signed written informed consent.

3.2.2 Exclusion criteria
• Inability or refusal to provide the informed consent;
• Concomitant neoplasia including hepatocellular carcinoma;
• Presence of ascites.

Liver stiffness measurements was performed using Fibroscan® (FibroScan 502 Echosens, Paris, France) within one month of the start of therapy (T0), and subsequently at their regular visits during the course of the antiviral therapy.
The scheme of visits during antiviral therapy was as follows: once during the first month of therapy, and then every three months during the whole duration of treatment and for one year following the termination of therapy. In accordance with international guidelines (18), the duration of antiviral therapy was not equal for all patients.
Sustained virological response (SVR) was defined as the absence of detectable HCV-RNA at 24 weeks’ follow up after the end of therapy.
The liver stiffness measurements were performed in the morning, following fasting from midnight, by placing an ultrasound transducer probe (M) of FibroScan® on the intercostal space at the area of the right liver lobe with patients lying in a supine position with maximal abduction of the right arm. They were carried out until 10 validated results had been obtained with a success rate of at least 90%. The median value of these 10 validated scores was considered the elastic modulus of the liver, and it was expressed in kilopascals (kPa). The measurements in kPa were converted into METAVIR score according to Ziol et al (85)
The protocol was approved by the local ethical committee of the Sant’Orsola-Malpighi Hospital, and carried out in compliance with the Helsinki declaration. Written informed consent was obtained from all enrolled patients.
3.3 Statistical analyses:

The result of LSM was expressed in kilopascal (kPa) as median value with an interquartile range (IQR). Continuous data were reported as mean ± SD. Student’s t-test and Chi square test were used to evaluate statistical significance of differences between groups. The Wilcoxon matched pairs signed-rank test was used to evaluate changes in LSM between baseline and end of follow-up. Stepwise multiple linear regression analysis was used to determine the factors associated with regression of liver stiffness. Receiver operating characteristic (ROC) curves were used to determine the reliability of the liver stiffness for predicting SVR. Statistical analyses were performed using SPSS version 21.0 software (SPSS Inc, Chicago, IL). A p value < 0.05 was considered statistically significant.

3.4 Results

A total of 72 patients were evaluated and 69 were enrolled. Their age ranged from 27 to 82 years. Table 1 shows the main demographic and clinical characteristics of enrolled patients at baseline. Twenty patients (29%) received the standard of care, that is pegylated interferon (PEG-IFN) plus ribavirin (RBV) while forty nine (71%) were treated with triple therapy that is telaprevir plus PEG-IFN and ribavirin (RBV). The mean duration of treatment was 37 weeks.
Table 1: Basal demographic and clinical characteristics of enrolled patients

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>69</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>58±11</td>
</tr>
<tr>
<td><strong>Gender, No. (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>37 (53.6)</td>
</tr>
<tr>
<td>Male</td>
<td>32 (46.4)</td>
</tr>
<tr>
<td><strong>Body Mass Index (Kg/m²)</strong></td>
<td>25±4 (17-41)</td>
</tr>
<tr>
<td><strong>HCV genotype, No. (%)</strong></td>
<td></td>
</tr>
<tr>
<td>G1a</td>
<td>4 (5.8)</td>
</tr>
<tr>
<td>G1b</td>
<td>32 (46.4)</td>
</tr>
<tr>
<td>G2</td>
<td>18 (26.1)</td>
</tr>
<tr>
<td>G3</td>
<td>11 (15.9)</td>
</tr>
<tr>
<td>G4</td>
<td>4 (5.8)</td>
</tr>
<tr>
<td><strong>Basal ALT (IU/L)</strong></td>
<td>80.4±49.1</td>
</tr>
<tr>
<td><strong>Treatment-naïve, No (%)</strong></td>
<td>41 (59.4)</td>
</tr>
<tr>
<td><strong>Type of treatment, No (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Standard of care (PEG-IFN/RBV)</td>
<td>20 (29)</td>
</tr>
<tr>
<td>Telaprevir plus standard of care</td>
<td>49 (71)</td>
</tr>
<tr>
<td><strong>Mean duration of treatment (weeks)</strong></td>
<td>37±14 (12-72)</td>
</tr>
</tbody>
</table>

At the end of the treatment period, 50 patients (72.5%) resulted sustained virological response (SVR) and the remaining 19 were non SVR (NSVR).

Liver stiffness and its changes

Before treatment (T0) LS values ranged from 3.0 to 48.0 kPa. Accordingly, 27 patients (39.1%) were classified in METAVIR fibrosis stage F0-1, 5 (7.2%) in F2, 12 (17.4%) in F3 and 25 (36.2%) in F4. As expected, at baseline patients obtaining SVR had a significantly lower LSM than NSVR patients (Table 2). Fibrosis evolution throughout the study duration is shown in table 2 and in figure 7. If compared to
baseline only SVR patients obtained a statistically significant reduction of LSM at the end of follow-up (P < 0.001), while no change was documented in NSVR.

Overall changes in METAVIR score were observed in 19 patients (27.5%): 7 showed regression of fibrosis by two METAVIR stages, 10 regression of one stage and two patients only showed fibrosis progression (Figure 8). Fibrosis regression was observed in more than one fourth (14/50) of SVR patients versus 16% (3/19) of the NSVR (figure 9). At the end of the study period, among SVR a significant reduction of LS was documented only in patients with baseline fibrosis score F4

Interestingly, at the end of the study period, that is twelve months after treatment discontinuation, all patients with F4 fibrosis (even non-responders) had shown a statistically significant decrease in LS (P=0.024) after the first 3 months of treatment (T3). However, this decrease was not predictive of SVR. Indeed the area under the ROC curve was 0.369 (CI %: 0.145-0.592; P= 0.265).

In our study population we did not found any significant association between fibrosis improvement and basal ALT levels, genotype, age, type of treatment and virologic response.
**Table 2**: changes in LSM from baseline through 12 months after treatment discontinuation in SVR and NSVR CHC patients.

<table>
<thead>
<tr>
<th>Time points</th>
<th>SVR (n=50)</th>
<th>Non-SVR (n=19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T0</strong></td>
<td>8.95 (3.0-35.3)</td>
<td>16.3 (3.8-48.0)</td>
<td>0.011</td>
</tr>
<tr>
<td><strong>T3</strong></td>
<td>7.9 (3.6-33.0)</td>
<td>16.0 (3.4-37.6)</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>EOT</strong></td>
<td>6.5 (3.2-45.0)</td>
<td>15.0 (4.9-46.4)</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>S1</strong></td>
<td>6.2 (3-39.2)</td>
<td>16.3 (4.4-48.0)</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>S3</strong></td>
<td>6.4 (3-45.7)</td>
<td>17.3 (4.0-39.0)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>S6</strong></td>
<td>6.1 (3.1-24.5)</td>
<td>17.3 (4.0-42.3)</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>S9</strong></td>
<td>6.5 (2.6-26)</td>
<td>16.5 (3.2-43.6)</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>S12</strong></td>
<td>5.8 (3.0-33.3)</td>
<td>16.0 (3.5-45.7)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

T0: baseline (before treatment); T3: third month of treatment; EOT: end of treatment; S: month(s) after the end of treatment.
Figure 7: Fibrosis evolution during the study duration in the 69 patients enrolled.

Figure 8: Changes in METAVIR score in all study population during the study.
3.5 Discussion and conclusions:

The clinical management and prognosis as well as the response to antiviral treatment of chronic hepatitis C are highly dependent on the extent of liver fibrosis. Quantification of liver fibrosis by non invasive means represents a major topic of research and discussion. Current data suggest that LS measurement could be used in many cases for quantifying fibrosis in patients with CHC.

The aim of the study was to analyze the effect of IFN-based antiviral therapy on evolution of liver fibrosis evaluated by means of Fibroscan in patients with chronic hepatitis C.

In our study, more than one third of patients had severe fibrosis (METAVIR stage F4) before treatment. In contrast, after treatment, most patients showed stabilization or regression of fibrosis (no progression). However, significant decrease in liver
stiffness was only detected in patients with sustained virologic response. Previous studies had also shown a significant decrease in liver stiffness values in sustained responders (87-91).

It is not surprising that before treatment LSM of patients subsequently obtaining SVR was significantly lower if compared to these of NSVR. Most studies have consistently shown that fibrosis is one of the most important predictor of virological response to IFN-based antiviral treatment in chronic hepatitis C.

As expected, SVR patients with higher initial LS (F4) showed the most significant regression of liver stiffness at the end of follow-up. It has been well documented that a sustained virological response is associated with improvement in both necroinflammatory scores and fibrosis (92-94, 97). Indeed, fibrosis has been wrongly considered an inactive tissue without regenerative potential for the organ affected. Within the last decade, this concept has changed and fibrosis is no longer considered irreversible, but the result of a continuous remodeling process and hence susceptible to interventions (95, 96). Evidence suggests that antiviral treatment may not only hinder the progression of liver fibrosis (98) but may actually reverse it (99). In particular, the anti-fibrogenic effects of IFN-alfa by decreasing the expression of transforming growth factors β1 mRNA and procollagen Type I mRNA in the liver has been consistently demonstrated (100).

Our study also showed that initial decrease in LSM, especially in patients with higher baseline fibrosis score is unlikely to predict an SVR. In addition no significant association was found between clinical or virological parameters and fibrosis improvement.

Even though the results of this prospective study conducted in patients with CHC treated with IFN-based antiviral therapy (standard and triple therapy) seem to confirm previous observations, the exact role of TE in monitoring response to treatment in HCV patients is still controversial. It has recently been shown that following therapeutic eradication of HCV, the predictive power of the viremic cut-off of 12 kPa is low as a consequence of liver remodelling and fibrosis reabsorption (101). Thus, at the moment liver biopsy still remains the only reliable approach to stage liver fibrosis following an SVR. However, the era of non invasive methods for evaluating liver fibrosis in now underway. Further studies are needed to delineate the most appropriate clinical scenarios for the LSM by Fibroscan in chronic hepatitis C and its role in monitoring the response to antiviral treatment.
References:


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