MEASURING LIVER STIFFNESS USING TRANSIENT ELASTOGRAPHY IN THE NON-INVASIVE ASSESSMENT OF CHRONIC HEPATITIS B

By

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ABSTRACT

Abstract of thesis entitled

Measuring liver stiffness using transient elastography in the non-invasive assessment of chronic hepatitis B

Submitted by James Yan Yue Fung

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Liver stiffness measurement using transient elastography has become recently available as a new non-invasive method to assess liver fibrosis in patients with chronic hepatitis B (CHB). The present thesis set out to determine the application of liver stiffness measurement in patients with CHB, including the long-term longitudinal changes and its prognostic value.

In a study of 1,268 CHB patients, liver stiffness correlated positively with routine liver biochemistry and hepatitis B virus (HBV) DNA levels, and negatively with albumin and platelet levels. Liver stiffness also correlated well with the different severity of liver diseases. The median liver stiffness in healthy subjects, occult hepatitis B, active hepatitis B, and end-stage cirrhosis were 4.6, 4.2, 8.7, and 33.8 kPa respectively.

Abstract

Using liver stiffness measurement to determine the prevalence of liver fibrosis in 951 CHB patients, 319 (34%) had severe fibrosis, with higher rates seen in older age groups, males, and in patients with higher ALT levels. However, the higher liver stiffness values observed in patients with higher ALT may be secondary to the affect of inflammation rather than fibrosis. A study on 29 patients with severe flare of ALT showed that liver stiffness was increased, with return to near normal levels by 6 months when the ALT levels were normalized. Liver biopsies on a subset of patients showed higher liver stiffness values at the time of flares than expected for the stage of fibrosis seen on histology. However, even mild to moderate elevation of ALT was shown to be associated with higher liver stiffness values independent of underlying liver fibrosis in a study of 38 patients.

For long-term prognosis, liver stiffness of ≥10 kPa was associated with a significantly increased risk of subsequent hepatocellular carcinoma development and mortality in 528 HBeAg-negative CHB patients. Longitudinal long-term follow-up also showed significant changes after 3 years in liver stiffness, although this was only observed in specific subsets of patients.

In conclusion, liver stiffness measurement with transient elastography is accurate in assessing liver fibrosis in CHB patients, and in determining long-term prognosis. However, elevation of ALT levels can significantly affect liver stiffness.

DEDICATION

This thesis is dedicated to Helen

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This thesis is composed of research projects of which there are many contributors, and I would like to express my utmost gratitude and appreciation for each of their contribution.

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CONTRIBUTIONS TO MEDICAL LITERATURE

Data from the studies embodied in the present thesis have been published in the literature listed below:

ORIGINAL ARTICLES

1. **Fung J**, Lai CL, Chan SC, But D, Seto WK, Cheng C, Wong DK, Lo CM, Fan ST, Yuen MF. Correlation of liver stiffness and histological features in healthy persons and in patients with occult hepatitis B, chronic active hepatitis B, or hepatitis B cirrhosis. Am J Gastroenterol. 2010 May;105(5):1116-22.

2. **Fung J**, Lai CL, Fong DY, Yuen JC, Wong DK, Yuen MF. Correlation of liver biochemistry with liver stiffness in chronic hepatitis B and development of a predictive model for liver fibrosis. Liver Int. 2008 Dec;28(10):1408-16.

3. **Fung J**, Lai CL, But D, Wong D, Cheung TK, Yuen MF.Prevalence of fibrosis and cirrhosis in chronic hepatitis B: implications for treatment and management. Am J Gastroenterol. 2008 Jun;103(6):1421-6.

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6. **Fung J,** Lai CL, Wong DK, Seto WK, Hung I, Yuen MF. Significant changes in liver stiffness measurements in patients with chronic hepatitis B: 3-year follow-up study. J Viral Hepat 2011 Jul;18(7):e200-5

7. **Fung J**, Lai CL, Cheng C, Wu R, Wong DK, Yuen MF. Mild-to-moderate elevation of alanine aminotransferase may increase liver stiffness measurement by transient elastography in patients with chronic hepatitis B. Am J Gastroenterol 2011. 106(3):492-6.

REVIEW ARTICLES

1. **Fung J**, Lai CL, Yuen MF. Clincial application of transient elastography (Fibroscan) in liver diseases. Hong Kong Medical Diary 2009;14(11):22-25

2. **Fung J,** Lai CL, Seto WK, Yuen MF. The use of transient elastography in the management of chronic hepatitis B. Hepatol Int 2011 Jun 22 [Epub ahead of print]

ABBREVIATIONS USED IN THESIS

AAR	aspartate aminotransferase to alanine aminotransferase ratio
AASLD	American Association for the Study of Liver Diseases
AFP	alpha-fetoprotein
ALP	alkaline phosphatase
ALT	alanine aminotransferase
Anti-HBc	antibody to hepatitis B core antigen
Anti-HBe	antibody to hepatitis B e-antigen
APASL	Asian-Pacific Association for the Study of the Liver
APGA	AST-Platelet-GGT-AFP index
API	age to platelet index
APRI	aspartate aminotransferase to platelet ratio index
AST	aspartate aminotransferase
AUROC	area under the receiver operating characteristic curve
BMI	body mass index
cccDNA	covalently close circular DNA
CHB	chronic hepatitis B
CT	computer tomography
EASL	European Association for the Study of the Liver
ECM	extra-cellular matrix
GGT	gamma-glutamyl-transpeptidase
HAI	histologic activity index

Abbreviations

HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e-antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBV DNA	hepatitis B virus DNA
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HSC	hepatic stellate cells
IQR	interquartile range
kPa	kilopascals
MRI	magnetic resonance imaging
NPV	negative predictive value
PBC	primary biliary cirrhosis
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PSC	primary sclerosing cholangitis
SVR	sustained virological response
TGF-β	transforming growth factor beta
ULN	upper limit of normal

Chapter I

CHRONIC HEPATITIS B INFECTION –

EPIDEMIOLOGY AND NATURAL HISTORY

Introduction

An estimated 400 million people worldwide are infected with the hepatitis B virus (HBV). The majority of people infected are from the Asia-Pacific or the sub-Saharan region. HBV infection is a significant health burden, since up to 40% of those infected may develop significant liver disease, including liver cirrhosis, liver decompensation, and hepatocellular carcinoma (HCC) (Lok & McMahon, 2009; McMahon, 2009). Currently, the best strategy to prevent HBV infection is by vaccination. Although the prevalence of chronic hepatitis B (CHB) has declined in regions where universal vaccination is available, the prevalence rate is still high for those who were born prior to the availability of universal vaccination, and for those people born in areas where vaccination is not readily available (Zacharakis et al., 2009; Zanetti, Van Damme, & Shouval, 2008).

After infection with HBV, the progression to chronic infection is dependent upon the age at which the virus is acquired. A younger age at the time of infection leads to higher rate of chronic infection. In newborns, the chronic carriage rate is approximately 90%, decreasing to 25% in preschool children, and declines to less than 3% by the time adolescence and young adulthood is reached (Okada, Kamiyama, Inomata, Imai, & Miyakawa, 1976; Stevens, Neurath, Beasley, & Szmuness, 1979). In Asia where HBV infection is endemic, perinatal transmission from mothers who are HBV carriers is the most common route of transmission, especially those with a high viral load (Burk, Hwang, Ho, Shafritz, & Beasley, 1994) or who are hepatitis B e-antigen (HBeAg)-positive (Okada et al., 1976; Stevens et al., 1979). The transmission of HBV occurs perinatally and can be prevented by the use of hepatitis B immunoglobulin and vaccination

schedule after birth. However a proportion may already have been infected *in utero* and are likely to become chronic carriers despite appropriate immunoprophylaxis (Tang, Hsu, Lin, Ni, & Chang, 1998). Babies can also be infected during early life by close contact with parents or siblings who are HBV carriers.

The different phases of CHB infection

The natural history of CHB can be described as 4 phases (Chen, 1993a, 1993b; Chu et al., 1985; Yim & Lok, 2006). There are key differences between ethnic groups, and this is shown in figure 1.1. The differences between Asians and Caucasian patients with CHB are important because of its impact on treatment decisions, especially after HBeAg seroconversion where a significant proportion of Asian patients will continue to have progressive disease (Lai & Yuen, 2007).

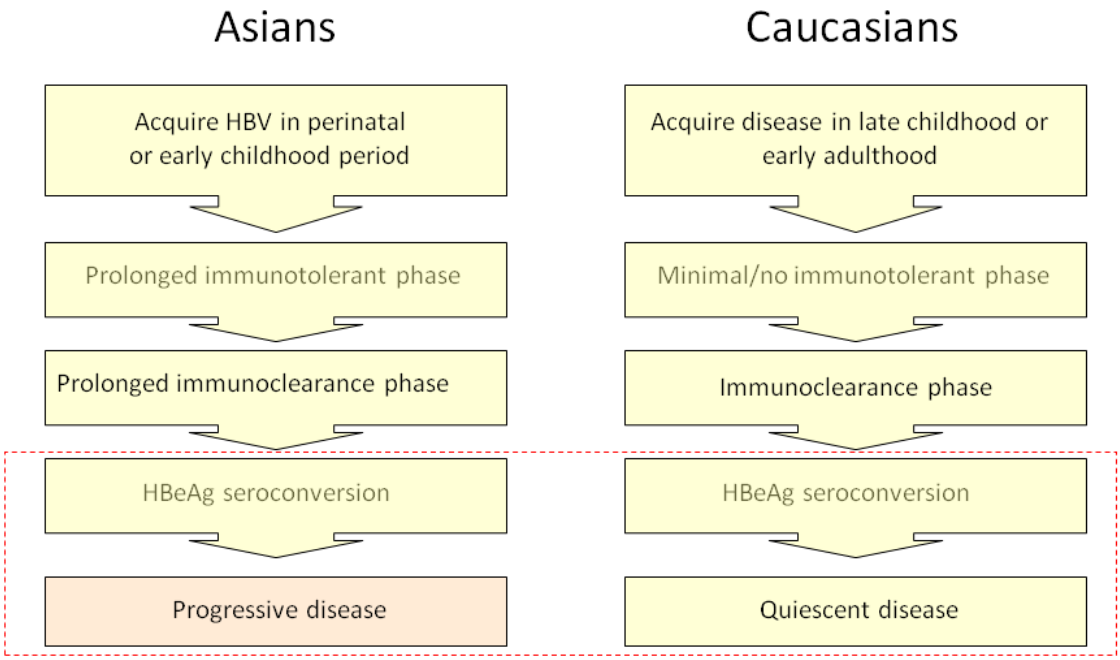

Figure 1.1 Difference in the natural history of chronic hepatitis B between Asian and Caucasian patients

The first phase is known as the immune-tolerant phase, and is characterized by HBeAg-positivity, very high levels of HBV DNA, normal alanine aminotransferase (ALT) levels, and minimal to no histological activity on liver biopsy. The hepatic injury is minimal because of the absence of host immune response against the virus. The immune-tolerant phase is characteristic of those people who acquire the virus during the perinatal or early childhood period, and is commonly observed in Asian countries. (Chen, 1993a, 1993b; Chu et al., 1985). In the Asian

CHB population, the median age of HBeAg seroconversion is around 30-35 years; therefore, the immune-tolerant phase may last for 20-30 years (Yuen et al., 2005). However, for people who acquire the virus in later childhood or during adulthood, the immune-tolerant phase is much shorter and may even be bypassed.

The second phase is known as the immune-clearance phase (also sometimes referred to as HBeAg-positive chronic hepatitis) and marks the end of immune-tolerance to HBV, with host immune response mounting an attack on the HBV-infected hepatocytes. The mechanism as to why the host immune response suddenly switches on and attempts to clear the virus is not known. As mentioned previously, those patients infected with HBV later in life may bypass the immune-tolerant phase and go directly into the immune-clearance phase. This phase is characterized by increased or fluctuating ALT levels and HBV DNA levels, with evidence of necro-inflammatory activity on liver biopsy. Host cellular response directed against HBV components, including hepatitis B core antigen (HBcAg) and HBeAg, leads to lysis of the infected hepatocytes (Tan, Koh, Goh, & Bertoletti, 2008; Tsai et al., 1992). It is often during this phase that liver fibrosis becomes evident by ongoing inflammatory and regenerative activity. If severe and prolonged, the fibrosis may progress to cirrhosis during this phase. Although patients may experience symptoms of acute hepatitis during this phase, a significant proportion of patients will remain asymptomatic.

Eventually, patients in the immune-clearance phase will undergo HBeAg seroconversion, with the development of antibody to HBeAg (anti-HBe). This marks the entry into the third phase of

CHB infection known as the low-replicative phase, or sometimes referred to as the inactive carrier phase. During this phase, there is normalization of the ALT level and the HBV DNA level is low or undetectable. Liver biopsy reveals minimal to no activity and the degree of fibrosis or the presence of cirrhosis usually reflects the damage that had accumulated during the immune-clearance phase. The prognosis of these patients at this stage therefore is largely dependent on the amount of fibrosis present. In patients with established cirrhosis, the changes in the liver architecture is likely to be permanent, and these patients will be at risk of develop HCC in the future, as well as decompensation in the setting of ongoing inflammatory activity.

Unfortunately, patients do not always remain in the inactive carrier phase (and some do not even have an inactive carrier phase, with ongoing active disease immediately after HBeAg seroconversion). A significant number of patients will enter the reactivation phase (fourth phase), with fluctuating ALT levels indicating active hepatitis. The HBV DNA becomes elevated and fluctuates throughout the course of this phase. There is usually significant amount of inflammatory activity on liver biopsy, and similar to the immune clearance phase, there may be evidence of significant fibrosis or cirrhosis observed. The course of this phase is usually refractory and patients usually require long-term antiviral therapy to reduce the risk of progression to cirrhosis and its complications, including decompensated liver disease and HCC. The exact mechanism for ongoing disease activity after loss of HBeAg is unclear, although mutations in the HBV have been implicated. Mutation in the precore region at position 1896 from G to A creates a stop codon which inhibits the production of HBeAg (Carman et al., 1989). However, the importance of this precore mutation in reactivation after loss of HBeAg is not known as this mutation has also been shown to occur in otherwise healthy inactive carriers with

anti-HBe (Okamoto et al., 1990). Other mutations in the core promoter region, including A1762T and G1764A, have also been shown to diminish HBeAg production (Okamoto et al., 1994). These mutations are associated with increase risk of subsequent development of cirrhosis and HCC (Kao, Chen, Lai, & Chen, 2003; Yang et al., 2008).

The best outcome for patients with CHB is the loss of hepatitis B surface antigen (HBsAg) with development of antibody to HBsAg (anti-HBs), which is also known as HBsAg seroconversion. However, even the loss of HBsAg does not signify complete cure from CHB, as low level of HBV DNA and covalently closed circular (ccc) DNA persists within the hepatocytes (Yuen, Wong et al., 2008) and may reactivate following loss of immune pressure from the host (such as that seen in patients receiving immunosuppressive therapy or chemotherapy) (Dervite, Hober, & Morel, 2001; Dhedin et al., 1998; Hui et al., 2006).

As described previously, and also shown in figure 1.2, most of the insult to the liver occurs during the immune-clearance phase and the reactivation phase, with evidence of necro-inflammatory activity within the liver leading to damage to the liver parenchymal tissue, and subsequent fibrosis and progression to cirrhosis. Therefore, it is important to identify those patients with significant fibrosis in order for these patients to receive timely antiviral therapy so that cirrhosis and decompensated liver disease can be prevented. By preventing the progression of fibrosis and cirrhosis with antiviral therapy, the risk of HCC may also be lowered (Liaw et al., 2004b; Yuen et al., 2007).

There are several important factors which have been shown to be associated with disease progression and the development of HCC. Within the last decade, the level of HBV DNA has been shown to be one of the most important factors associated with long-term outcome (C. J. Chen et al., 2006; G. Chen et al., 2006; Iloeje et al., 2006; Martinot-Peignoux et al., 2002; Yuan, Yuen, Ka-Ho Wong, Sablon, & Lai, 2005; Yuen et al., 2009). Higher levels of HBV DNA have been associated with complications of CHB including cirrhosis and HCC, without a safe cut-off level in which complications does not occur. More recently, the levels of HBsAg have been shown to be significantly different in different phase of CHB infection (Chan et al., 2010; Jaroszewicz et al., 2010; T. Nguyen et al., 2010). In addition to levels of HBV DNA and HBsAg, hepatitis B genotype may also affect the natural history of CHB infection, with genotype C being associated with poorer outcomes, including advanced fibrosis and cirrhosis, and the development of HCC (Chan, Wong, Tse et al., 2009; Kao, 2007; Kao, Chen, Lai, & Chen, 2000, 2004; Ni et al., 2004; Yu et al., 2005). The next chapter will discuss in detail the pathogenesis and assessment of liver fibrosis.

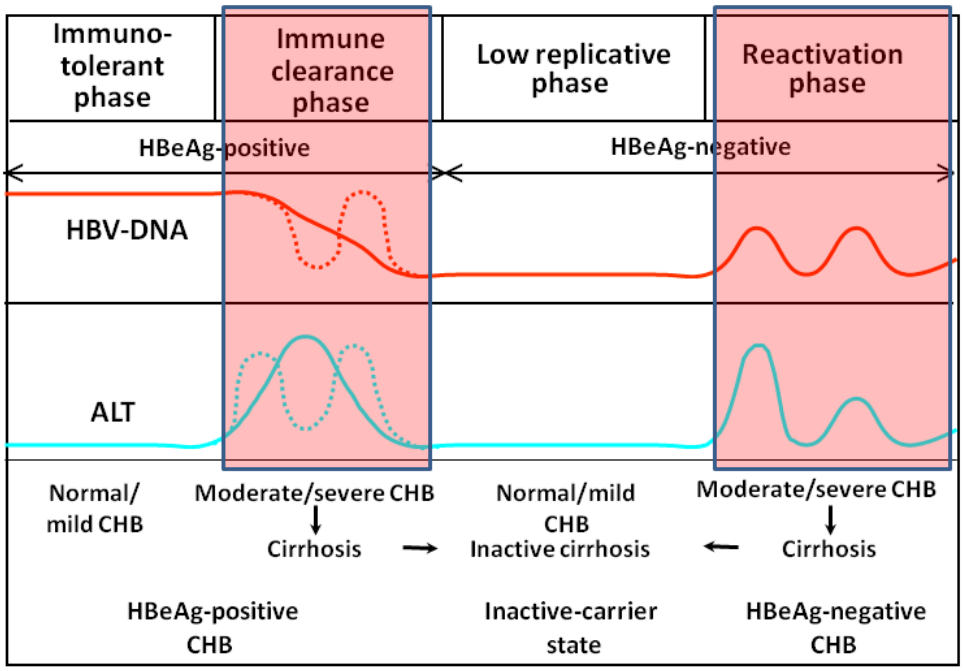

Figure 1.2 The characteristics of the four major phases of chronic hepatitis B infection

Chapter II

LIVER FIBROSIS -

PATHOGENESIS AND ASSESSMENT

Introduction

Liver fibrosis occurs as a result from chronic damage to the liver. Almost any condition which causes inflammation to the liver may cause fibrosis, including chronic viral hepatitis, such as hepatitis B and C. Liver fibrosis is an insidious process and the patients remain asymptomatic during its development. The majority of liver-related morbidity and mortality occurs after the development of cirrhosis, with evidence of decompensation or the development of HCC. The progression of fibrosis to cirrhosis usually is a slow process, occurring after a period of 15-20 years, although more rapid progression may be seen in those with severe injury as seen in drug hepatotoxicity, recurrent hepatitis C virus (HCV) and HBV infection after liver transplantation (Schluger et al., 1996), and HCV/HIV co-infection (Bonnard et al., 2007). Acute hepatitis will activate the mechanism of fibrogenesis, but in order for significant fibrosis to accumulate, sustained chronic injury is required, such as that observed in CHB. In spite of the ongoing insult to the liver for decades from CHB, the majority of patients will have slow progression of fibrosis largely due to the regenerative capability of the liver.

Pathogenesis of liver fibrosis

The process of liver fibrosis can be described as a wound-healing response to ongoing insult to the liver. There is excessive accumulation of extra-cellular matrix (ECM) proteins which leads to distortion of the liver architecture with formation of fibrous scar tissue. The ECM proteins include 3 large families of proteins: the collagens, glycoproteins, and proteoglycans. During an acute episode of hepatitis, such as in acute hepatitis B, there is regeneration of parenchymal cells which replaces the injured or necrotic cells. The deposition of ECM proteins during this acute

episode is usually limited. However, with ongoing injury, such as that seen in CHB (during the immune-clearance phase or the reactivation phase), the regeneration of parenchymal cells 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.

Hepatic stellate cells

In the liver, hepatic stellate cells (HSC) comprise approximately 15% of the total resident liver cells, and play a pivotal role in fibrogenesis. They are located in the parasinusoidal space and are the principal storage site for retinoids in the normal liver. The HSCs are normally quiescent and produces only small amounts of ECM proteins for the formation of the basement membrane (Geerts, 2001). Activation of HSC consists of the initiation and perpetuation phase, which is followed by a resolution phase when the liver injury resolves (Friedman, 2004). The initiation stage is characterized by changes in gene expression and phenotype that makes the HSC responsive to other cytokines and stimuli. This is usually from paracrine stimulation secondary to changes in the surrounding ECM and damaged hepatocytes leading to necrosis and apoptosis with subsequent generation of oxidative stress, which can activate HSC (Novo et al., 2006). The activated HSC transforms into myofibroblast-like cells to become the main ECM-producing cells (Marra, 1999; Milani et al., 1990). The perpetuation phase of the HSC involves six major changes in cellular behavior, namely proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, and retinoid loss. The net effect of these changes is the increase in accumulation of

ECM and replacement of normal matrix with scar tissue. Each one of these changes are governed by a complex interplay of cytokines and growth factors involving both autocrine and paracrine stimulation.

An example of such signaling factor includes transforming growth factor beta (TGF-β), a fibrogenic cytokine secreted by not only HSCs, but also by Kupffer cells, hepatocytes, and platelets (Friedman, 1999; Gressner, Weiskirchen, Breitkopf, & Dooley, 2002; Shek & Benyon, 2004). TGF-β is the most potent stimulus for the production of collagen I and other ECM components by the HSC. Another cytokine include platelet derived growth factor (PDGF). PDGF is the predominant mitogenic cytokine for HSCs secreted mainly by Kupffer cells, and expression of PDGF is up-regulated during activation of the HSC (Pinzani, Gesualdo, Sabbah, & Abboud, 1989). The HSCs can migrate towards the sites of injury through the process of chemotaxis by cytokines such as PDGF (Ikeda et al., 1999; Kinnman et al., 2000), monocyte chemoattractant protein-1 (Marra et al., 1999), and CXCR3 ligands (Bonacchi et al., 2001). Secretion of large amounts of ECM occurs with accumulation of HSCs at the repair sites.

One of the important behaviors of HSC is that of matrix degradation, as fibrosis represents the balance between matrix production and degradation. The resorption of excess matrix in patients with chronic liver diseases such as CHB allows for reversal of hepatic dysfunction and fibrosis. However, degradation of matrix may have a deleterious effect on liver function if it is subsequently replaced by scar tissue and not normal liver tissue. The key enzymes in matrix degradation are the family of matrix-metalloproteinase (MMP) which specifically degrades

collagens and non-collagenous substrates. The MMP activity can be inactivated by binding to tissue inhibitors of MMP (TIMP) (Iredale, 2001). TIMP-1 is also anti-apoptotic towards HSC, and its expression at the time of liver injury will maintain or increase the population of activated HSCs by preventing their clearance (Murphy et al., 2002). The importance of the major cytokines such as TGF-β and enzymes such as those from the family of MMP and TIMP extends to their potential use as specific biomarkers of fibrinogenesis, which will be discussed in further detail in the following sections.

During resolution of liver injury, HSC may either revert back to a quiescent phenotype (Gaca et al., 2003; Guyot, Combe, Balabaud, Bioulac-Sage, & Desmouliere, 2007) or undergo clearance by the process of apoptosis. Although the HSC remains one of the major contributors towards the total fibrogenic cell population during liver injury, there is increasing evidence that the fibrogenic cells are derived not only from the resident HSC. Other sites and cellular components which also contribute to this fibrogenesis include portal fibroblasts (Beaussier et al., 2007; Jhandier, Kruglov, Lavoie, Sevigny, & Dranoff, 2005; Kinnman & Housset, 2002; Magness, Bataller, Yang, & Brenner, 2004), circulating fibrocytes (Kisseleva et al., 2006), bone marrow (Forbes et al., 2004), hepatocytes and cholangiocytes (Kalluri & Neilson, 2003; Zeisberg et al., 2007). Figure 2.1 shows a schematic representation of HSC activation, the major changes in cellular behavior, and the different cellular contributors to the fibrogenic process.

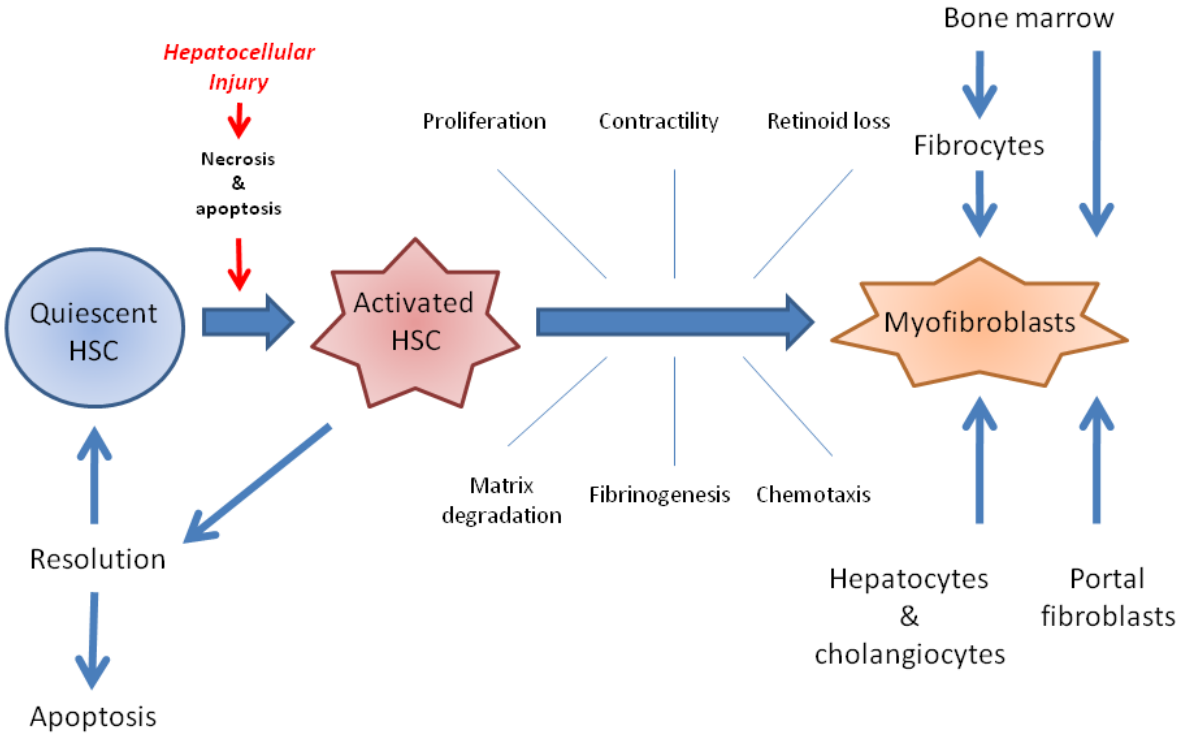

Figure 2.1 The role of hepatic stellate cells in liver fibrosis and contribution of other cellular components

Assessment of liver fibrosis

In the clinical setting, it is important for clinicians to determine the amount, stage, or severity of fibrosis for many reasons. Firstly, the assessment of fibrosis can guide treatment decisions and identify eligible patients for treatment. In fact, the current major regional treatment guidelines all advocate the use of liver biopsy to select patients who may benefit from antiviral therapy. The American Association for the Study of Liver Diseases (AASLD) (Lok & McMahon, 2009) and

Asian Pacific Association for the Study of the Liver (APASL) (Liaw, Leung et al., 2008) guidelines suggest consideration for liver biopsy in those patients over the age of 40 years, with borderline or mildly elevated ALT on serial testing, and HBV DNA >20,000 IU/mL or ≥2,000 IU/mL in HBeAg-positive and HBeAg-negative patients respectively. The European Association for the Study of the Liver (EASL) (EASL, 2009) treatment guidelines include histological grade and stage as one of their 3 criteria for treatment (the other 2 being serum HBV DNA and ALT levels). Those with moderate-to-severe necro-inflammatory activity and/or fibrosis would be recommended for antiviral therapy.

Secondly, assessment of fibrosis can allow the clinicians to gauge the underlying disease severity, and identify those patients who already have established cirrhosis, but otherwise remain asymptomatic. This is important as patients with established cirrhosis should be considered for screening/surveillance for HCC with 6-monthly α-fetoprotein (AFP) measurements and ultrasound imaging (McMahon et al., 2000; Yuen et al., 2000; B. H. Zhang, Yang, & Tang, 2004). Those patients with cirrhosis should also be considered for screening with upper endoscopy for the presence of oesophageal varices, gastric varices, and portal hypertensive gastropathy. In addition, those with established cirrhosis should be eligible for antiviral therapy with a lower HBV DNA threshold irrespective of the ALT levels (Lok & McMahon, 2009).

Assessment for liver fibrosis in CHB is also important as it may have prognostic significance. Those patients with advanced fibrosis or cirrhosis are more likely to have poorer long-term outcome, and closer monitoring of these patients is warranted (Park et al., 2007). In addition,

interval fibrosis assessment can also determine treatment response to see whether there is any improvement in the degree of fibrosis after commencing antiviral therapy.

Methods of assessing liver fibrosis

There are currently 3 major methods used in the clinical setting to assess liver fibrosis: liver biopsy, biochemical markers, and liver stiffness measurement. The latter two methods are non-invasive in nature, of which liver stiffness measurement will be discussed on its own in the following chapter.

Liver biopsy

Liver biopsy is currently the gold standard in the assessment of liver fibrosis. In addition to being able to stage the degree of fibrosis, liver biopsy also provides information regarding the degree of inflammatory activity in the liver, and also has useful diagnostic applications in a wide range of liver diseases where histology is often needed to confirm the diagnosis (for example, in autoimmune hepatitis and primary biliary cirrhosis). Despite being the gold standard, liver biopsy remains an imperfect benchmark for several important reasons. Many patients are reluctant to undergo a liver biopsy because of the perceived risk of the procedure. Significant complications requiring hospital admissions or prolonged hospital stays have been estimated to occur in 1-5% of patients after liver biopsy, whereas the mortality rate for liver biopsy has been estimated to be 1:1000 to 1:10,000 (McGill, Rakela, Zinsmeister, & Ott, 1990; Piccinino, Sagnelli, Pasquale, & Giusti, 1986). Also, the biopsy needle only samples a very tiny portion of

the liver (estimated to be approximately 1/50,000) (Bravo, Sheth, & Chopra, 2001), leading to problems with significant sampling errors as the disease may not always affect the liver in a homogenous pattern (Abdi, Millan, & Mezey, 1979; Bedossa, Dargere, & Paradis, 2003). This is further compounded by the fact that the rate of sampling error and variability in interpretation is dependent on the length of the specimen obtained and the degree of fragmentation (Colloredo, Guido, Sonzogni, & Leandro, 2003; Regev et al., 2002). Figure 2.2 illustrates the example of sampling error depending on the location of the biopsy needle, showing a significant discrepancy between the severities of fibrosis between the 2 samples within the same liver. To increase the accuracy of liver biopsy, an adequate specimen length must be obtained. It has been estimated that for liver biopsy to approach 100% accuracy, a 40mm specimen length is required. However, this is rarely obtained, and the majority of the biopsy length obtained (approximately 80%) fall into the 5-15mm category, resulting in an accuracy rate of approximately 80%. Finally, the interpretation of the histology by pathologists are subjected to both inter- and intra- observer variability, further compounding the reduced accuracy of liver biopsy in the assessment of fibrosis (Goldin et al., 1996; Soloway, Baggenstoss, Schoenfield, & Summerskill, 1971; Westin, Lagging, Wejstal, Norkrans, & Dhillon, 1999).

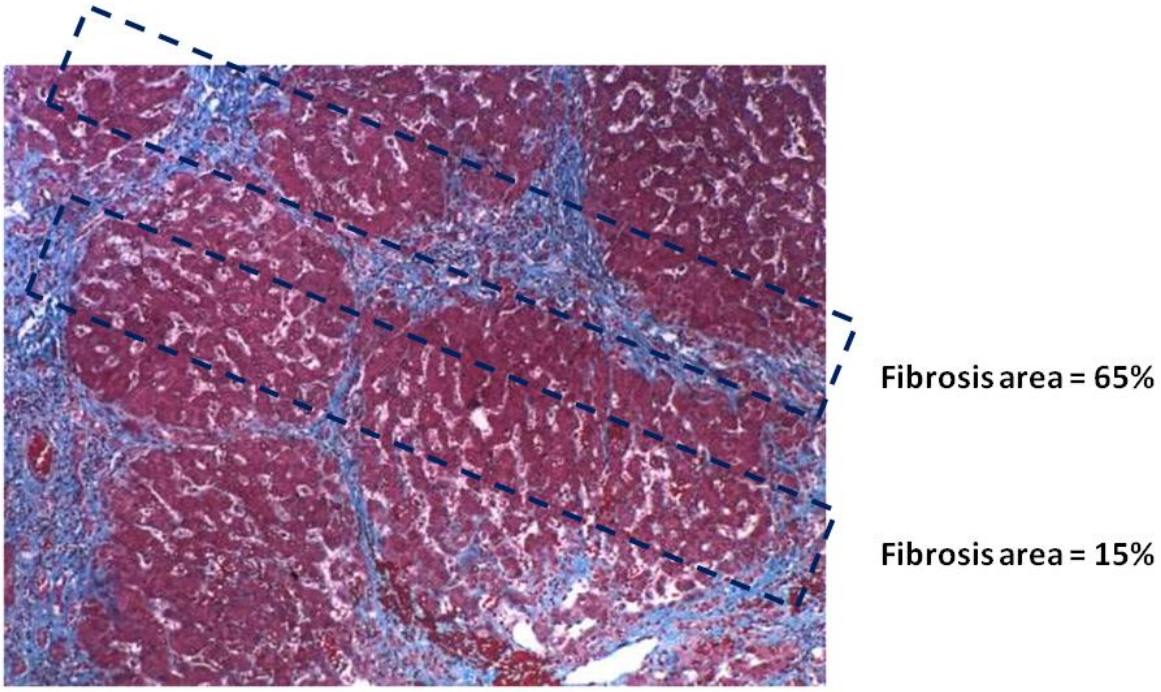

Figure 2.2 Potential sampling error using liver biopsy in the assessment of liver fibrosis

Scoring systems of fibrosis

The severity of liver fibrosis can be described in stages, ranging from no fibrosis to frank cirrhosis. There are many grading and staging systems which can be used to describe the histo-pathological process of the liver, and some of these may be disease-specific. The first histological classification was develop in 1968 which characterised the terms chronic persistent hepatitis and chronic aggressive hepatitis, distinguished by the severity of piecemeal necrosis, inflammation and structural remodelling of the liver (De Groote et al., 1968). The Histology

Activity Index (HAI) score was developed in 1981, which comprises of 3 categories for necro-inflammation and 1 for fibrosis, with points given for severity of the lesion in each category, and the sum total making up the final score (Knodell et al., 1981). Since then, there have been many newer staging systems, most of which reports the grade and stage, although the criteria for scoring may be different. For example, the METAVIR scoring system for chronic hepatitis C employs a two-letter and two-number system (Bedossa & Poynard, 1996). For inflammatory activity, A0 = no activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity. For fibrosis staging, this is shown in table 2.1.

Table 2.1 Fibrosis staging using the METAVIR system (Bedossa & Poynard, 1996)

Score	Description
0	No fibrosis
1	Stellate enlargement of portal tract but without septa formation
2	Enlargement of portal tract with rare septa formation
3	Numerous septa without cirrhosis
4	Cirrhosis

The other commonly used system is the modified Knodell HAI score, which is also known as the Ishak system, allows for a maximum score of 18 for grading of inflammation, and 6 for fibrosis. The scoring for fibrosis under the Ishak is decsribed in table 2.2.

Table 2.2 Fibrosis staging using the Ishak modified HAI system (Ishak et al., 1995)

Score	Description
0	No fibrosis
1	Fibrous expansion of some portal areas, with or without short fibrous septa
2	Fibrous expansion of most portal areas, with or without short fibrous septa
3	Fibrous expansion of most portal areas, with occasional portal to portal bridging
4	Fibrous expansion of portal areas with marked bridging as well as portal-central
5	Marked bridging with occasional nodules
6	Cirrhosis

Given the numerous scoring systems available, and the heterogeneity of their components, it is unlikely that any single system is adequate. Furthermore, the scoring system is categorical, assessing the architectural changes within the liver, rather than quantifying the amount of actual fibrosis (or collagen deposition). The numerical increments of fibrosis stage also may be misleading as the interpreters may assume a linear increase in the amount of fibrosis within the liver with increasing fibrosis score. Therefore, the current staging system compounds the short-comings of liver biopsies as a gold standard for assessing fibrosis.

Biochemical markers of fibrosis

Over the recent years there has been an exponential increase in the interest in non-invasively assessing liver fibrosis through the use of biochemical markers. These biomarkers can be

grouped broadly into 4 categories: biomarkers of liver cell injury, biomarkers of inflammation, biomarkers of fibrogenesis, and biomarkers of fibrosis and ECM-turnover (see figure 2.3).

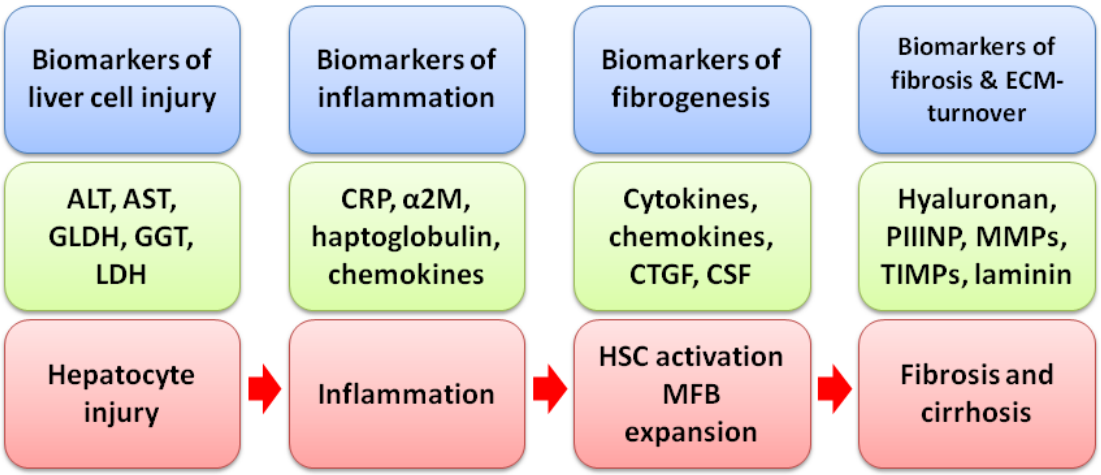

Figure 2.3 Different groups of biomarkers for the non-invasive assessment of liver fibrosis. ALT = alanine aminotransferase; AST = aspartate aminotransferase; GLDH = glutamate dehydrogenase; GGT = gamma-glutamyl transpeptidase; LDH = lactic dehydrogenase; CRP = C-reactive protein; α2M = α2-macroglobulin; CTGF = connective tissue growth factor; CSF = colony stimulating factor; HSC = hepatic stellate cells; MFB = Myofibroblasts; ECM = extracellular matrix; PIIINP = procollagen III N-terminal propeptide; MMP = matrix metalloproteinase; TIMP = tissue inhibitors of metalloproteinase

These biomarkers can also be classified into Class I and Class II, some of which has been described in the previous chapter on liver fibrosis. Class I biomarkers are derived from ECM turnover and reflect the activity of both the fibrogenic and fibrinolytic process that is associated with ECM remodeling. However, these do not indicate the extent of connective tissue deposition, and therefore, despite that most of these biomarkers are associated with fibrogenic activity, their use as biomarkers to assess liver fibrosis as been purely hypothesis-driven. Moreover, these tests are often not readily available as routine tests in many hospital laboratories, and are frequently costly. Examples of Class I biomarkers include procollagen, TIMP, laminin, and hyaluronic acid (HA). In contrast, Class II biomarkers include commonly readily available tests such as aspartate aminotransferase (AST), platelets, bilirubin, and γ-glutamyl transpeptidase (GGT). Although these biomarkers have either indirect or no direct patho-biochemical connection with the process of fibrogenesis, they have been shown to be strongly correlated with the degree of underlying fibrosis.

Both class I and II biomarkers individually are not sensitive or specific enough to stage liver fibrosis when used alone. Therefore, the majority of tests employ the use of a panel of biomarkers which combines a number of these biomarkers together to increase the accuracy of these tests. A list of examples of these panels of tests is shown in table 2.3, and numerous others have been developed to compete for higher accuracy in predicting liver fibrosis.

Table 2.3 Various scoring panels using a combination of biomarkers for the assessment of liver fibrosis

Tests	Components	Disease
PGAA index	Prothrombin time, GGT, apo-A1, α2-macroglobulin	Alcohol
Forns tests	Age, platelets, GGT, cholesterol	HCV
Pohl score	AST/ALT ratio, platelet count	HCV
APRI	AST, platelets	HCV
Fibrotest	GGT, haptoglobin, bilirubin, apolipoprotein A, a2-macroglobulin	HCV/HBV
FIB-4	Platelet, AST, ALT, age	HCV/HIV
HepaScore	Bilirubin, GGT, hyaluronan, α2-macroglobulin, age, gender	HCV
FPI	Age, AST, cholesterol, HOMA-IR	HCV

PGAA index (Naveau, Poynard, Benattar, Bedossa, & Chaput, 1994; Poynard et al., 1991); Forns tests (Forns et al., 2002); Pohl score (Pohl et al., 2001); APRI (Wai et al., 2003); Fibrotest (Imbert-Bismut et al., 2001); FIB-4 (Sterling et al., 2006); Hepascore (Adams et al., 2005); FPI = fibrosis probability index (Sud et al., 2004)

To use these tests, a logarithmic formula is usually derived using these individual components. Examples of such formulae are shown below:

- **Forns** = 7.811 - 3.131 x ln platelets (G/L) + 0.781 x ln GGT (U/L) + 3.467 x ln age (years) – 0.014 x cholesterol (g/L) (Forns et al., 2002)
- **Hepascore** = y/(1+y); y = exp(-4.185818 – (0.0249 x age) + (0.7464 x 1 if male, 0 if female) + (1.0039 x α2 macro) + (0.0302 x hyaluronate) + (0.0691 x bilirubin) – (0.0012 x GGT)) (Adams et al., 2005)
- **APRI** = AST/upper limit of normal x 100/platelet (10^9/L) (Wai et al., 2003)
- **Fibrometer** = -0.007 x platelets (g/L) – 0.049 x prothrombin time (%) + 0.012 x AST (U/mL) + 0.005 x α2 macro (mg/dL) + 0.021 x hyaluronate (mg/L) – 0.270 x urea (mmol/L) + 0.027 x age (years) + 3.718 (Cales et al., 2005)
- **APGA**: log (index) = 1.44 + 0.1490 log (GGT) + 0.3308 log (AST) – 0.5846 log (platelets) + 0.1148 log (AFP + 1) (Fung, Lai, Fong et al., 2008)

As can be seen, many of these formulas are complicated and cumbersome, and include the use of biomarkers that are not in routine use. Therefore, there is limited application in the clinical setting for daily care of patients using these complicated indices. The reported sensitivity and specificity for these tests have been variable. Those with higher sensitivity usually have lower specificity, and vice versa. In addition, many of these biomarkers are not liver-specific, and fibrosis and inflammation occurring in other organs may affect the results. Despite the recent increase in the number of panels and scoring systems becoming available

for the non-invasive assessment of liver fibrosis, including the commercial availability of at least one of these panels (Imbert-Bismut et al., 2001), they have not been widely accepted into clinical practice worldwide. In addition to identifying better markers using proteomics, novel serum markers may be potentially discovered by analyzing liver transcriptomes in histological specimens. However, the identification of a single novel marker remains difficult as most gene products are not shed into the circulation, and are not related to fibrogenesis. Currently, preliminary works have been on patients with chronic hepatitis C (Asselah et al., 2005; Takahara et al., 2008).

Clearly, other modalities of non-invasive test for liver fibrosis is needed which does not require the use of biomarkers. Over the past 5 years, major advances have been made with liver stiffness measurement using transient elastography as an alternative non-invasive tool for assessing liver fibrosis.

Chapter III

NON-INVASIVE ASSESSMENT OF LIVER FIBROSIS BY LIVER STIFFNESS MEASUREMENT USING TRANSIENT ELASTOGRAPHY (FIBROSCAN®)

Introduction

Liver stiffness measurement by transient elastography has become widely available both in Europe and Asian-Pacific countries over the last 5 years as a new non-invasive method for assessing liver fibrosis. In fact, the visco-elastic characteristics of liver tissue were already observed in an early study looking at pig hepatic tissue by the combination of optical method and indentation technique (B. C. Wang, Wang, Yan, & Liu, 1992). The elastic properties of the human liver were later estimated by measuring the internal displacement and strain (Yamashita & Kubota, 1994, 1995). Another study found that the elastic properties of hepatic haemangiomas were harder than the background liver tissue (Emelianov, Rubin, Lubinski, Skovoroda, & O'Donnell, 1998), demonstrating the difference in elastic properties of normal and pathological tissue. The notion that liver fibrosis may lead to changes in the mechanical properties of the liver was shown earlier almost a decade ago. A study on 19 fresh human liver samples and 1 focal nodular hyperplasia sample (obtained intra-operatively) using cyclic compression-relaxation showed that the elastic modulus generally increased with the fibrosis grade, with values in the order of several hundreds to thousands of Pascals (Yeh et al., 2002). There was a significant correlation shown between the fibrosis score and the elastic modulus, suggesting that elasticity imaging of the liver may be able to predict stages of fibrosis.

The term “Elastography” refers to imaging methods which assess tissue elasticity. All elastographic methods share a common 3 step methodology:

1. Generating a low frequency vibration within the tissue to induce shear stress
2. Imaging of the tissue to analyze the stress that is induced

3. Calculating the tissue stiffness from the above

Several elastographic techniques have been described, including remote elastography, dynamic elastography, static elastography, real-time elastography, and transient elastography. Remote elastography employs the induction of low frequency vibrations in tissues by acoustic radiation force remotely, in combination with ultrasonic imaging (Nightingale, Palmeri, Nightingale, & Trahey, 2001). Dynamic elastography using magnetic resonance imaging (MRI) have been described, but requires long acquisition times coupled with high costs for the MRI scan (Muthupillai et al., 1995; Yin et al., 2007). Static elastography employs a uniform compression at the body surface to cause deformation in the tissue (Hall, Zhu, & Spalding, 2003; Ophir, Cespedes, Ponnekanti, Yazdi, & Li, 1991). However, because of the location of the liver, it is not feasible to place it under controlled compression. Real-time elastography can image the physical property of tissue using conventional ultrasound probes, and results have been promising in assessing liver fibrosis (Friedrich-Rust et al., 2007). By far the most established elastography method in assessing liver fibrosis is that of transient elastography.

Principles of Transient Elastography

Liver stiffness can be measured by a physical quantity known as Young's modulus, which is expressed in pressure units of Pascals (Pa). The definition of Young's modulus (E) is a simple ratio between the applied stress (s) and the induced strain (e) (Sarvazyan, 2001):

$$E = \frac{s}{e}$$

This concept can be best described in the following schematic diagram (figure 3.1).

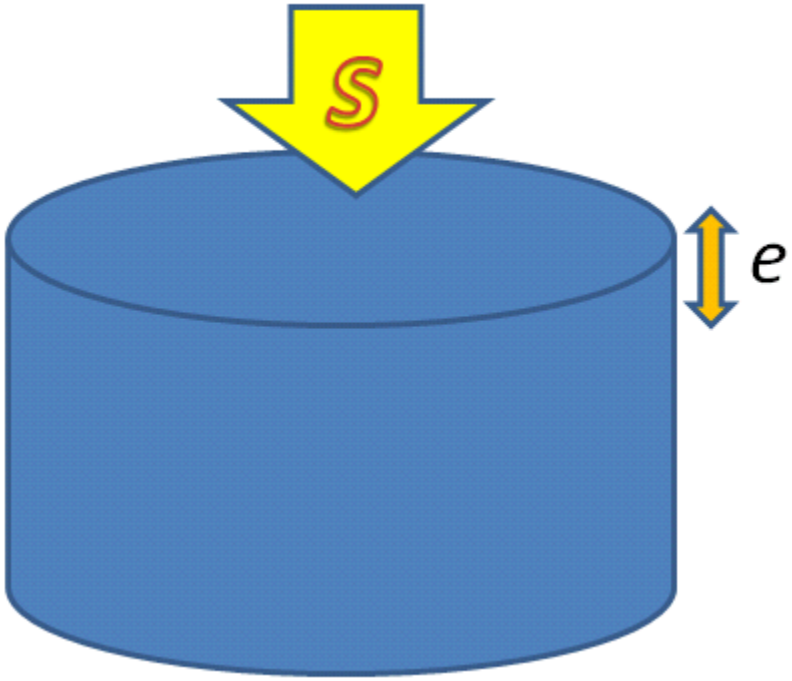

Figure 3.1 Deformation of a soft solid under an externally applied stress. S=stress, e= strain.

An external stress (s) when applied to tissue will induce a deformation or strain (e) within the tissue. If the tissue is harder, then the induced strain (e) is smaller, resulting in a higher Young's modulus compared to softer tissue (Duck, 1990). The density of tissue within the body is very close to the density of water (1000 kg/m^3) and remains relatively constant. However, the elasticity or stiffness of the tissue may vary significantly depending on the underlying pathological state (Skovoroda et al., 1995).

In addition to a static compression, a transient impulse can be induced in the tissue through more complex mechanical methods. Through this method, 2 types of waves can be mechanically induced. The first type of waves is called compression waves, and these propagate at very high velocity (1500 m/s) by compressing successively layers of tissue. The second type of wave is called shear waves. Compared to compression waves, these are much slower, propagating at speeds of 1 to 10 m/s by creating a tangential sliding force between the tissue layers. In solid matter, the stiffness is dependent on both the compression modulus (λ) and the shear modulus (μ). However, in soft tissues, the shear modulus is sufficient to estimate the stiffness. The shear modulus can be derived from the following formula where ρ is the density of tissue (1000 kg/m^3) and Vs is the shear wave propagation velocity:

$$\mu = \rho V_s^2$$

The elasticity of the soft tissue and the shear wave propagation speed can be linked using the following simple formula (Royer & Dieulesaint, 2000):

$$E = 3\rho V_s^2 \text{ or } E = 3\mu$$

There are several advantages in the use of transient waves and vibrations. Firstly, transient waves are less sensitive to boundary conditions, and less prone to artifacts. In addition, the acquisition time is extremely short, allowing for measurements from the liver being possible given that the liver moves constantly with respiration.

Operation of Transient Elastography

Like all elastographic methods, transient elastography also employs the 3 step methods as described previously. A vibrating piston is used to create a mechanical wave of low frequency and low amplitude. This creates a shear wave which is propagated through the liver tissue (figure 3.2)

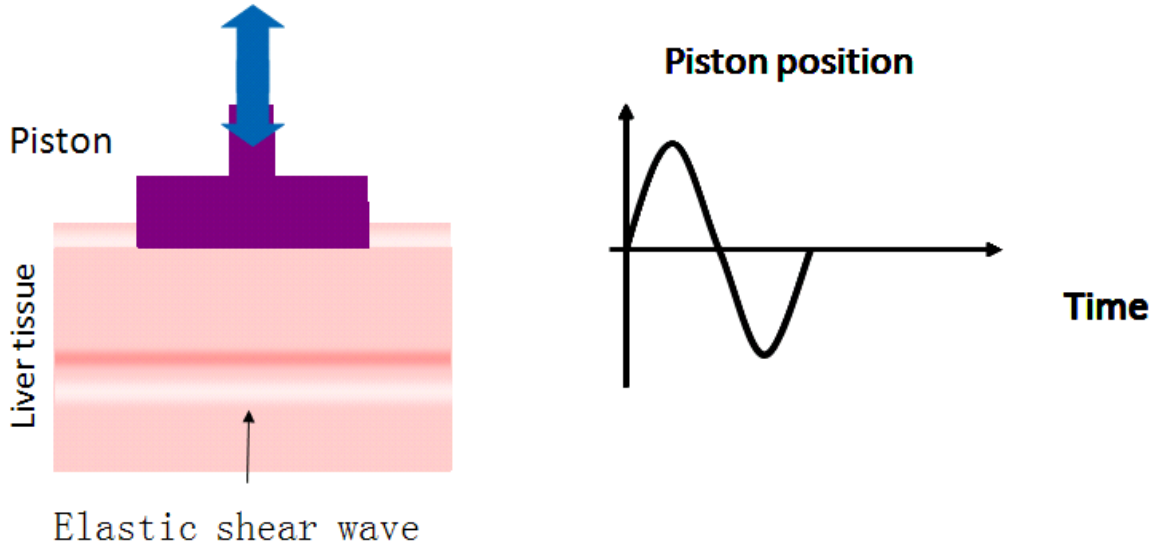

Figure 3.2 Propagation of the shear wave though the liver tissue from the vibrating piston

The shear wave propagation is detected using a transducer that is located at the tip of the probe (figure 3.3)

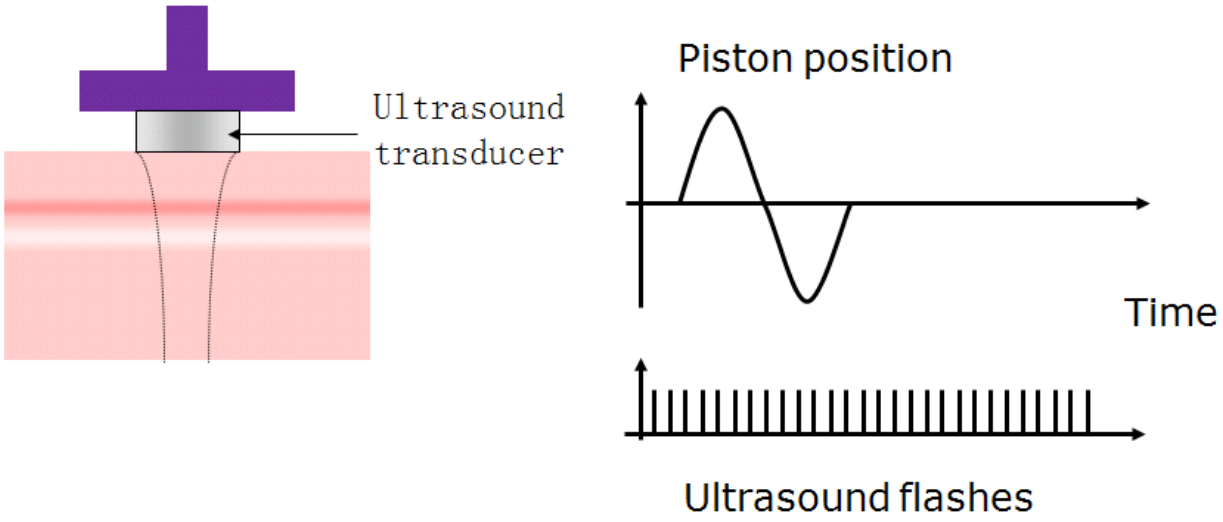

Figure 3.3 Ultrasound transducer located at the tip of the probe can detect the perturbation created by the vibrating piston

Comparisons with consecutive ultrasound signals are then performed to map out the mechanical perturbation that was induced by the vibrating piston. A typical example of the image mapped out by ultrasound showing the perturbation rate is shown in figure 3.4.

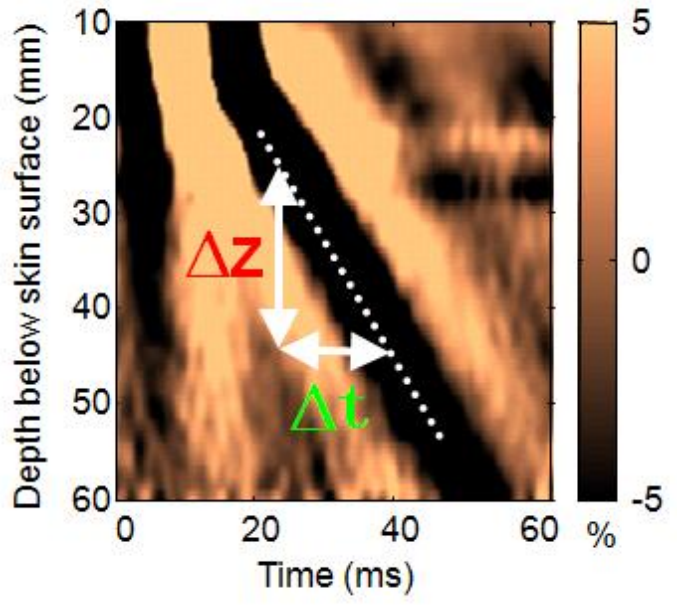

Figure 3.4 Typical image pattern characterizing the perturbation rate

From this image, the velocity of the shear wave can be calculated using the formula:

$$V_s = \frac{\Delta Z}{\Delta t}$$

And the liver stiffness calculation can be derived using the following formula as previously described:

$$E = 3\rho V_s^2$$

The following diagram shows an example of the different perturbation rates equating to different degrees of underlying fibrosis (figure 3.5).

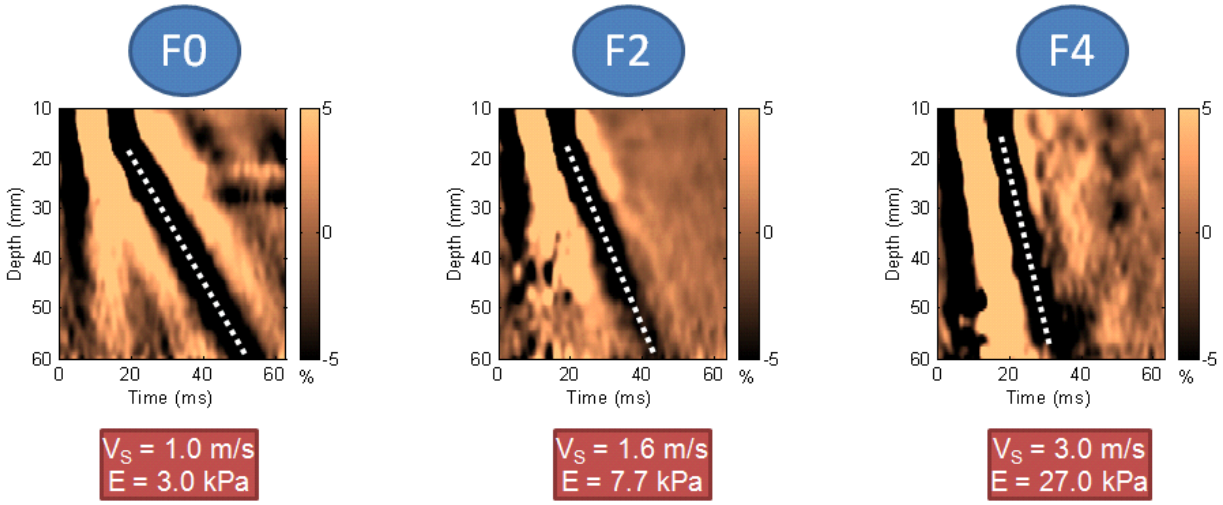

Figure 3.5 Different perturbation rates and shear wave velocity corresponding to different stages of liver fibrosis

As can be seen in the above figure, higher shear wave velocity corresponds to higher liver stiffness, which corresponds to a higher stage of fibrosis.

Hardware in Transient Elastography

The Fibroscan® (Echosens, Paris, France) is the currently available hardware used for liver stiffness measurement by transient elastography, and is the hardware used for liver stiffness measurement in the studies for 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 3.6). The probe is connected to a main

computer and control unit (figure 3.7) which calculates the liver stiffness values and displays the results on a monitor.

Figure 3.6 The probe of the Fibroscan

A total of 256 radiofrequency lines are acquired at a repetition frequency of 4000 Hz during the propagation of the low-frequency elastic wave through the liver. 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.

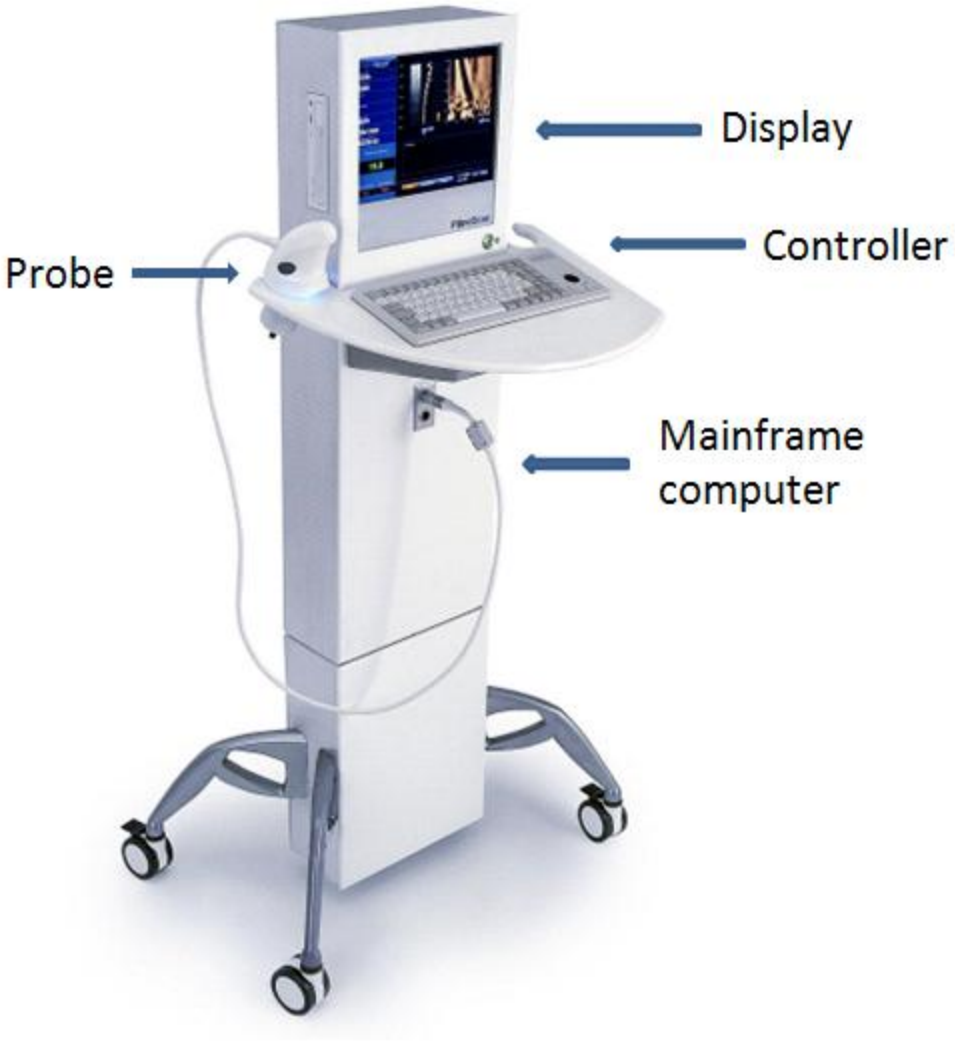

Figure 3.7 The different components of the Fibroscan

Acquisition of liver stiffness

The position of the patient during transient elastography is essentially the same position that is assumed for liver biopsy. The patient is lying in the dorsal decubitus position with the right arm in full abduction. Coupling gel is applied to the tip of the probe, and the probe is then placed over the right lobe of the liver between the intercostal spaces. To measure the shear wave velocity accurately, the probe is placed at a plane that is both vertically and horizontally perpendicular to the skin surface. An ultrasonic time-motion image is used to locate a portion of liver that is approximately 6 cm in thickness and free of large vascular structures which may alter the shear wave velocity. Once a suitable portion of the liver is identified, the operator then presses the button on the probe to begin an acquisition. At this point, the patient will feel a sensation similar to a light poke which is non-painful at the skin surface as the vibrating piston fires off an impulse. The computer mainframe then analyzes the data acquired by the ultrasound probe to determine the velocity of the shear wave, and to calculate the liver stiffness. The results are displayed on the screen, of which an example is shown in figure 3.8. If the acquisition was unsuccessful, an invalid result is given. At least 10 valid acquisitions are required for each patient, and the median liver stiffness out of the total valid acquisitions will be used as the final liver stiffness result. The median value is used so that the results will not be skewed by a single spuriously low or high liver stiffness value. The range of liver stiffness value is from a minimum of 2.5 kPa to a maximum reading of 75.0 kPa. The success rate is calculated by the number of successful acquisition divided by the total number of acquisitions (including the number of invalid acquisitions). A success rate of at least 50% is needed before the final results can be deemed as valid, although the exact accepted cut-off used is usually stated. Further criteria to improve the accuracy of liver stiffness measurements were adopted as more validation studies

were performed subsequent to the initial validation studies. In addition to the success rate, the interquartile range (IQR) of the valid acquisitions to final liver stiffness score (the median score) ratio should also be less than 0.30, although once again, the exact cut-off adopted is usually stated. The entire procedure usually takes less than 5 minutes although in more difficult patients, a longer procedure time is required.

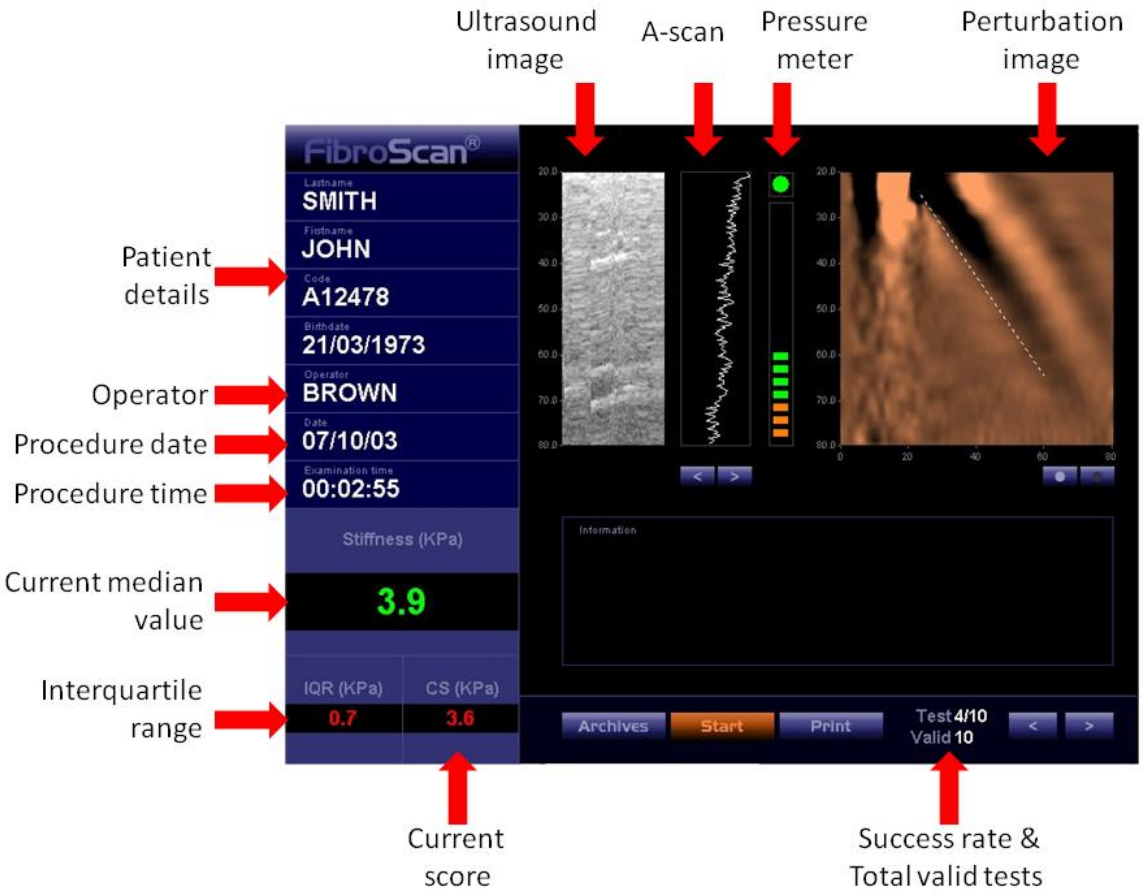

Figure 3.8 Screenshot after acquisition of liver stiffness measurement using the Fibroscan

Advantages of liver stiffness measurement

There are several major advantages of transient elastography compared with liver biopsy. Firstly, liver stiffness measurement 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 transient elastography. Secondly, 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. Thirdly, the patient does not require hospital admission or correction of clotting profile (such as those on anticoagulation therapy or with underlying coagulopathy). Fourthly, the liver stiffness measurement is obtained using $1/500^{th}$ volume of the total liver, compared to $1/50{,}000^{th}$ that is examined by liver biopsy (Bravo et al., 2001). Therefore, a much larger area of the liver is surveyed, making sampling error less likely.

Disadvantages of liver stiffness measurement

Despite the morbidity associated with liver biopsy, it remains the gold standard in assessing fibrosis, and the benchmark which all other non-invasive tests are validated against. One disadvantage of transient elastography is that fact that it is useful only for the evaluation of liver fibrosis, and in contrast to a biopsy, it provides no diagnostic information. A liver biopsy can often provide information as to the underlying cause of the fibrosis, such as autoimmune hepatitis, haemochromatosis, Wilson's disease, primary biliary cirrhosis, steatohepatitis, and a wide range of other liver diseases. In addition, transient elastography does not provide information as to the degree of activity or inflammation that is present in the liver. In contrast, a

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 CHB.

Accuracy of liver stiffness measurements

Most of the initial validation studies on transient elastography were performed in patients with chronic hepatitis C, likely due to the fact that the technology originated from France where HCV is prevalent. One of the earlier studies validating liver stiffness measurements in chronic hepatitis C was performed on 251 patients, showing a higher liver stiffness with increasing fibrosis according to the METAVIR scoring system (Ziol et al., 2005). From 2005 onwards, there have been many more studies validating the diagnostic accuracy of transient elastography in the assessment of liver fibrosis. In addition to chronic hepatitis C, liver stiffness measurement has also been validated in other liver diseases including fatty liver disease (Friedrich-Rust et al., 2010; Yoneda et al., 2008), PBC and PSC (Corpechot et al., 2006), and alcoholic liver disease (Nahon et al., 2008).

Most of the studies validating transient elastography have reported the accuracy using the area under the receiver operating characteristic curve (AUROC), with an area of 1 representing a perfect test. Values of 0.90-1 and 0.80-0.90 are considered to be excellent and good tests respectively. The receiver operating characteristic curve is a plot of (1-specificity) of the test in question on the x-axis against its sensitivity on the y-axis for all possible cut-off points. The optimal cut-offs described in various studies corresponding to the different fibrosis stages are often the values imparting the highest combination of sensitivity and specificity. The positive

predictive value and the negative predictive value of the test will be dependent on the selected optimal cut-off value.

A meta-analysis of 50 studies confirmed the accuracy of transient elastography in diagnosing liver fibrosis (Friedrich-Rust et al., 2008). The performance was best when used to differentiate cirrhosis versus no cirrhosis with a mean AUROC of 94%, and an adjusted AUROC of 99%. The mean AUROC for the diagnosis of significant fibrosis and severe fibrosis was 0.84 and 0.89 respectively.

For CHB, there has been much less studies validating transient elastography. A study of 173 CHB patients with liver biopsies confirmed the accuracy of liver stiffness measurement with an AUROC of 0.81, 0.93 and 0.93 for fibrosis stages F≥2, F≥3, and F=4 respectively. The optimal cut-off values derived from the study on CHB patients for fibrosis stages F≥2, F≥3, and F=4 were 7.2, 8.1, and 11.0 kPa respectively (Marcellin et al., 2009). Shortly after the introduction of transient elastography in 2005, the only validation study for CHB patients was published in abstract form, using a cut-off value of 10.3 kPa for cirrhosis. This cut-off value was adopted for the initial studies of this thesis (Marcellin et al., 2005).

For obese patients, recent availability of the XL probe (with a new 2.5 MHz ultrasonic transducer and new electrodynamic transducer) analyzing a deeper area of 3.5 to 7.5cm may improve the diagnostic accuracy and yield in this group of patients. The liver stiffness values

obtained with this probe may differ with that obtained by the standard probe; therefore validation studies are required for newer probes (V. de Ledinghen et al., 2010).

Outline of the thesis

At the time of commencing this thesis in the final quarter of 2005, transient elastography had been made available for a few months only. There had been no publications in peer reviewed journals on the use of transient elastography in CHB.

The aims of the studies presented in this thesis are to:

1) Correlate the routine biochemical findings with liver stiffness measurements in CHB patients
2) Determine the prevalence of significant fibrosis in a population of CHB patients
3) Determine the impact of acute hepatitis B flares on liver stiffness measurements
4) Determine the effects of mild-to-moderate elevations of liver transaminase on liver stiffness measurements
5) Determine the different levels of liver stiffness in patients with a wide spectrum of hepatitis B-related liver disease
6) Determine the prognostic value of liver stiffness measurements for HCC in patients with CHB
7) Determine the value of liver stiffness in longitudinal long-term follow-up of patients with CHB

Chapter IV

CORRELATION OF LIVER BIOCHEMISTRY WITH LIVER STIFFNESS IN CHRONIC HEPATITIS B AND DEVELOPMENT OF A PREDICTIVE MODEL FOR LIVER FIBROSIS

Introduction

There is an increasing demand for non-invasive tests to assess liver fibrosis because of the reluctance of patients to undergo liver biopsy. As previously mentioned, some of these non-invasive tests have focused on using markers of collagen matrix synthesis and degradation such as hyaluronic acid and procollagen III (Rosenberg et al., 2004). Others have used readily available laboratory markers alone and in combination including the AST/ALT ratio (AAR) (E. Giannini et al., 1999; E. Giannini et al., 2003; E. G. Giannini et al., 2006; Imperiale, Said, Cummings, & Born, 2000), globulin (Schmilovitz-Weiss et al., 2006), and AST/platelet ratio index (APRI) (Snyder et al., 2006; Wai et al., 2003). In addition, several panels of assays are now commercially available for the assessment of fibrosis using a combination of markers including α2-macroglobulin, haptoglobin, apolipoprotein A1, GGT, ALT and total bilirubin (Imbert-Bismut et al., 2001; Poynard et al., 2004).

Measuring liver stiffness by transient elastography has been shown to correlate well with liver fibrosis in CHB (Marcellin et al., 2005). Given that transient elastography is a relatively new technology, there have been no previous studies correlating patient demographic factors and liver biochemistry with liver stiffness measurements in CHB.

The aims of this study were to correlate liver stiffness measurements with patient demographics and laboratory parameters, and to assess the potential predictive value of these laboratory parameters for fibrosis and cirrhosis by using liver stiffness measurements.

Patients and Methods

Patients

Transient elastography was performed in CHB patients who attended the Hepatitis Clinic, Queen Mary Hospital during the period of January 2006 to December 2006. CHB infection was defined as being HBsAg positive for 6 months or more. Those with HCV co-infection, autoimmune liver disease, Wilson's disease, PBC or significant alcohol intake (>20g/day) were excluded. Patient demographics and laboratory parameters were recorded at the time of liver stiffness measurement, including age, sex, bilirubin, ALT, albumin, AFP, HBsAg, HBeAg, anti-HBe, and serum HBV DNA levels. HBV DNA was measured using the COBAS Taqman® HBV DNA assay, with a lower and upper limit of detection of 12 IU/mL and 1.1 x 10^8 IU/mL respectively [Roche Diagnostics, Branchburg, NJ].

Measurement of liver stiffness

Transient elastography was performed using the Fibroscan® (EchoSens, Paris, France). The method of liver stiffness measurements is described in the previous chapter. The liver stiffness scores are expressed in units of kilopascals (kPa). Liver stiffness scores of >8.1 and >10.3 kPa were used as cut-off values for the presence of significant liver fibrosis and liver cirrhosis respectively, in accordance with a previous study on CHB patients (Marcellin et al., 2005).

Statistical analysis

All statistical analyses were performed using SPSS version 14.0 (SPSS Inc, Chicago, IL), Statistical Analysis System (SAS) 2.4.1, and Stata/SE 9.2. The Mann-Whitney U test was used for continuous variables with skewed distribution. Chi-squared test was used for categorical variables, and Fischer's test where appropriate. The comparison of different median values of more than two groups was made by Kruskal-Wallis test. The correlation co-efficient was calculated using Spearman test. A p-value of <0.05 was considered statistically significant.

To derive a new index using commonly measured laboratory markers, the study sample was randomly split into a training set and a validation set. All variables including liver stiffness measurements underwent logarithmic transformation for better model fit. The sequence of variables in order of their associations with liver stiffness (co-efficient path) was determined using L1 regularized regression. The AUROC were calculated for each number of variables used for the prediction of fibrosis and cirrhosis, and the number of variables used was determined as the one that additional variables would not give a relatively higher accuracy.

A new index score was then developed and the optimal cut-off value was determined as the value with the highest sensitivity and specificity. Using the new index score on the validation set, the AUROC, sensitivity, specificity, predictive values and likelihood ratios were calculated.

The new index score was compared to pre-existing non-invasive indices including the AST/platelet ratio index (APRI), age to platelet index (API) and the AST/ALT ratio (AAR). The APRI was calculated using ([AST/ upper limit of normal (ULN)]/platelet count [x10^9/L]) x 100 (Wai et al., 2003). The AAR was calculated using AST/ALT (E. Giannini et al., 1999). The API was scored using the sum of age and platelet count scores as defined by age (years)<30=0; 30-39=1; 40-49=2; 50-59=3; 60-69=4; ≥70=5 and platelet count (x10^9/L)≥225=0; 200-224=1, 175-199=2; 150-174=3; 125-149=4; <125=5 (Poynard & Bedossa, 1997).

Results

Patient demographics.

A total of 1268 unselected CHB patients underwent liver stiffness measurement. Twenty seven (2%) patients had failed liver stiffness measurements due to obesity or excess overlying adipose tissue, and were excluded. Another forty-five (4%) patients were excluded due to success rates of less than 50% in liver stiffness measurement to obtain the required 10 valid measurements. In total, 1196 CHB patients were included in the final analysis. The demographics of these 1196 patients are listed in table 4.1.

The comparison between patient demographic factors including age, sex and HBeAg status and median score of liver stiffness are listed in Table 4.2. Both male gender and HBeAg positivity were associated with a higher median liver stiffness. After the age of 35 years, increasing age was associated with an increasing median liver stiffness.

Table 4.1 Patient demographic and laboratory data

Parameters	Value
Total patients	1268
Failed Fibroscan	27 (2%)
Suboptimal scans (success rates <50%)	45 (4%)
Final analysis	1196
Age (years)	43 (17-88)
Male sex	789 (66%)
HBeAg-negative disease	930 (78%)
Liver stiffness (kPa)	6.6 (1.5-75.0)
Success rates (%)	100 (50-100)
Bilirubin (µmol/L)	11 (1-172)
ALT (U/L)	33 (8-962)
Albumin (g/L)	44 (22-51)
Alpha-fetoprotein (ng/mL)	3 (0-300)
HBV DNA (IU/mL)	29500 (12-1.1 x 10^8)

* All continuous variables are expressed as median levels

Table 4.2 Patient demographics and liver stiffness

Parameters	**N**	**Liver stiffness (median, kPa)**	**P value**
Sex			**<0.001**
Male	789	7.1 (1.5-75.0)	
Female	407	5.9 (2.8-67.8)	
Age (years)			**<0.001***
≤25	66	5.9 (3.3-16.8)	
			0.580
26-35	244	5.9 (1.5-40.9)	
			0.001
36-45	398	6.3 (1.5-57.3)	
			<0.001
46-55	372	7.4 (2.8-75.0)	
			0.056
56-65	92	8.7 (3.0-67.8)	
			0.015
>65	24	12.2 (5.7-29.9)	
HBeAg			**0.03**
Positive	266	6.8 (1.5-75.0)	
Negative	930	6.6 (1.5-62.8)	

* for overall trend

Laboratory parameters and liver stiffness.

Using the Spearman correlation test, the correlation co-efficient for serum liver biochemistry and HBV DNA in relation to liver stiffness measurements are shown in table 4.3. The laboratory markers with the highest correlation co-efficients were GGT, AST, ALT and platelets (0.495, 0.506, 0.455 and -0.496 respectively, all $p<0.001$). The comparison of liver stiffness between patients with different serum ALT levels is shown in figure 4.1. There was a positive correlation with ALT levels and liver stiffness measurements from <0.5 x ULN up to 5 x ULN. The comparison of liver stiffness with different levels of serum albumin, bilirubin and AFP are shown in figure 4.2. There was positive correlation seen with bilirubin and AFP levels with liver stiffness, and an inverse correlation seen with albumin level and liver stiffness.

Table 4.3. Correlation of liver stiffness measurements with routine liver function tests and viral load

Laboratory parameters	**Correlation coefficient**	**P value**
Bilirubin	0.194	<0.001
ALP	0.267	<0.001
GGT	0.495	<0.001
AST	0.506	<0.001
ALT	0.455	<0.001
Albumin	-0.218	<0.001
Globulin	0.185	<0.001
Platelets	-0.495	<0.001
Alpha fetoprotein	0.317	<0.001
HBV DNA	0.138	0.002

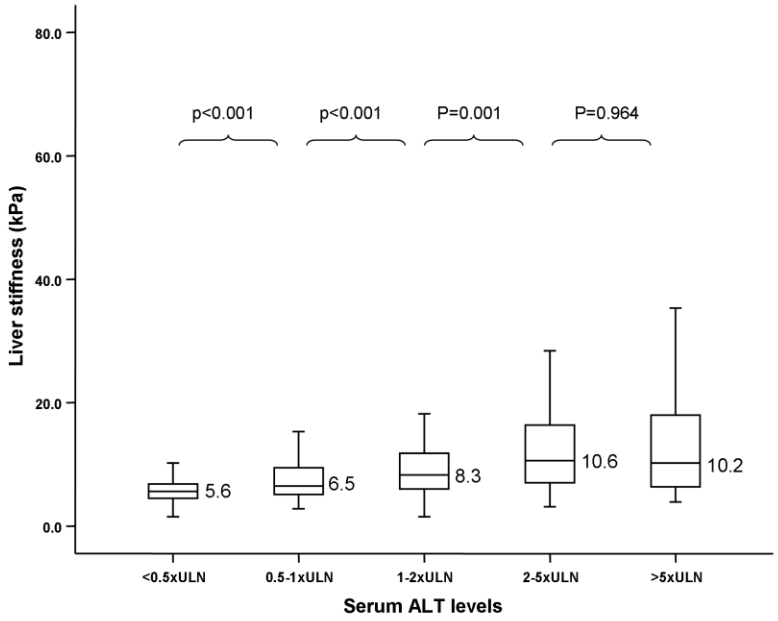

Figure 4.1. Liver stiffness measurements and different ALT levels. Box plots show median values with 25th and 75th percentiles.

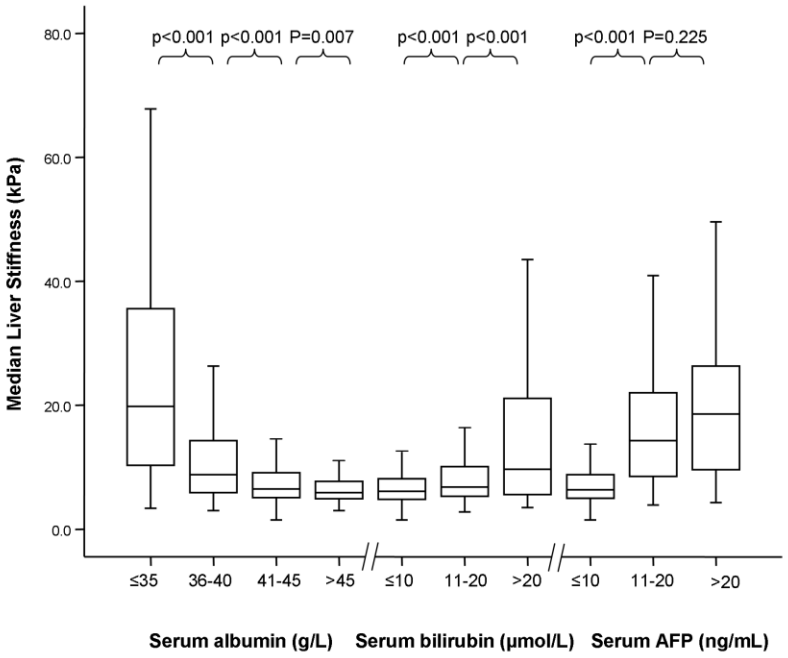

Figure 4.2. Liver stiffness measurements and serum albumin, serum bilirubin and serum AFP levels. Box plots show median values with 25th and 75th percentiles. AFP=alpha fetoprotein.

Non-invasive index of fibrosis and cirrhosis

A total of 265 patients from the current cohort had results available in all 13 variables chosen for calculation of predictive index (age, sex, platelet, AST, ALT, GGT, AFP, albumin, globulin, bilirubin, ALP, HBV DNA, and HBeAg) at the time of liver stiffness measurements. The characteristics of these patients are listed in table 4.4. These were split randomly into a training set of 139 patients and validation set of 126 patients. After using 4 of the 13 variables (platelets, AST, GGT and AFP), additional variables did not improve the accuracy significantly, as shown in figure 4.3. Using stepwise regression, the APGA (AST/Platelet/GGT/AFP) index was derived as follows: log(index) = 1.44 + 0.1490log(GGT) + 0.3308log (AST) – 0.5846log(platelets) + 0.1148log (AFP+1). Table 4.5 shows the accuracy results of both these groups in predicting significant fibrosis and cirrhosis. Using an optimal cutoff value of 6.9 for significant fibrosis, the sensitivity was 82% with a specificity of 69%, with a negative predictive value (NPV) of 91% in the validation set. For cirrhosis, an optimal cutoff of 8.9 was associated with a sensitivity of 64% and specificity of 89% with a NPV of 92%.

The AUROC for predicting significant fibrosis were both 0.85 in the training and validation set respectively. The AUROC for predicting cirrhosis using a cut-off of 10.3 kPa were 0.89 and 0.85 in the training and validation group respectively. Using a higher liver stiffness measurement of 12 kPa as a cutoff for cirrhosis as suggested by some validation studies for chronic liver diseases (Castera et al., 2005; Foucher, Chanteloup et al., 2006; Gomez-Dominguez et al., 2006), a higher AUROC of 0.91 was achieved, with a specificity of 92% and a NPV of 96% in predicting cirrhosis.

Table 4.4 Characteristics of patients included in the predictive model

Parameters	**All**	**Training**	**Validation**
Number	265	139	126
Age (years)	42 (19-73)	42 (19-73)	42 (19-71)
Male sex	170 (64%)	91 (34%)	79 (30%)
HBeAg (-)	169 (64%)	90 (34%)	79 (30%)
Fibrosis	90 (34%)	56 (21%)	13 (13%)
Cirrhosis	63 (24%)	41 (15%)	22 (8%)
Bilirubin (µmol/L)	11 (2-172)	11 (3-46)	12 (2-172)
ALT (U/L)	31 (8-393)	33 (9-386)	29 (8-393)
Albumin (g/L)	44 (27-51)	44 (27-50)	44 (35-51)
Alpha-fetoprotein	4 (0-167)	4 (0-167)	4 (0-45)
HBV DNA (IU/ml)	35,600	25,130	57,250
	(12-1.1 x 10^8)	(12-1.1 x 10^8)	(12-1.1 x 10^8)
ALP	67 (27-300)	68 (32-300)	66 (27-209)
AST	25 (13-274)	26 (14-271)	25 (13-274)
GGT	24 (8-370)	27 (10-235)	23 (8-370)
Globulin	33 (26-53)	33 (26-53)	33 (26-48)
Platelets	213 (54-505)	211 (54-405)	216 (73-505)

* Continuous variables are expressed as median values

Figure 4.3 Area under the receiver operating characteristics curve at each step. Step 1 to 14 as listed in their order: platelets, AST, GGT, AFP, albumin, gender, globulin, bilirubin, ALP, HBV DNA, ALT, age, and HBeAg status

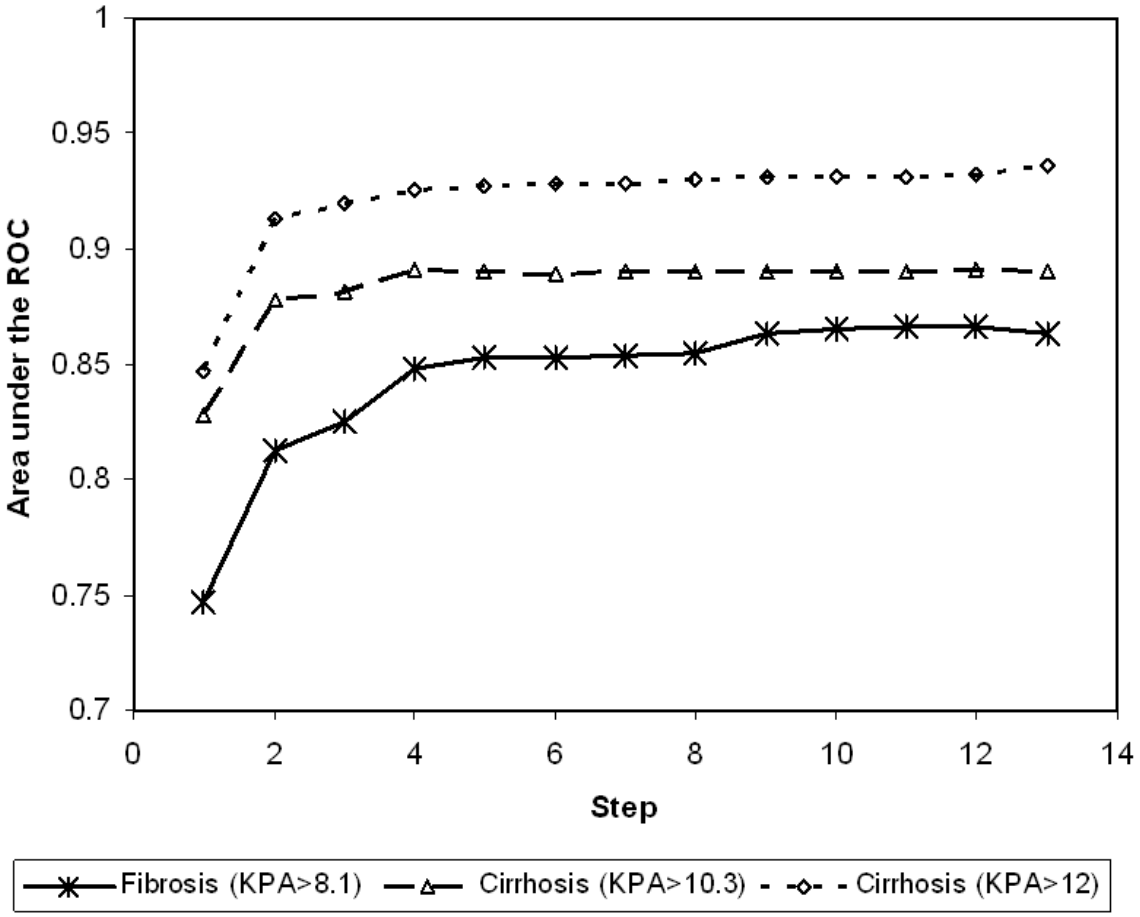

Table 4.5 Accuracy results in predicting fibrosis and cirrhosis in training and validation subjects using the APGA index

	Fibrosis	**Cirrhosis**
Training		
AUC Training	0.85 (0.78, 0.92)	0.89 (0.82, 0.96)
Optimal cutoff (kPa)	6.8687	8.9283
Sensitivity	82.1% (69.9%, 91.1%)	78% (62.4%, 89.4%)
Specificity	73.5% (62.7%, 82.6%)	85.7% (77.2%, 92%)
Positive likelihood ratio	3.1 (2.12, 4.52)	5.46 (3.28, 9.11)
Negative likelihood ratio	0.24 (0.14, 0.43)	0.26 (0.14, 0.46)
Positive predictive value	67.6% (55.2%, 78.5%)	69.6% (54.2%, 82.3%)
Negative predictive value	85.9% (75.6%, 93%)	90.3% (82.4%, 95.5%)
Validation		
AUC Training	0.85 (0.78, 0.92)	0.85 (0.76, 0.93)
Sensitivity	82.4% (65.5%, 93.2%)	63.6% (40.7%, 82.8%)
Specificity	68.5% (58%, 77.8%)	88.5% (80.7%, 93.9%)
Positive likelihood ratio	2.61 (1.86, 3.67)	5.52 (2.97, 10.2)
Negative likelihood ratio	0.26 (0.12, 0.54)	0.41 (0.24, 0.72)
Positive predictive value	49.1% (35.6%, 62.7%)	53.8% (33.4%, 73.4%)
Negative predictive value	91.3% (82%, 96.7%)	92% (84.8%, 96.5%)

Figure 4.4 and 4.5 show the AUROC for predicting significant fibrosis and cirrhosis respectively for both training and validation sets, comparing the APGA index with APRI, API and AAR. The AUROC comparing the APGA index with APRI, API and AAR is shown in table 4.6. The AUROC for the APGA index was higher compared to that of the APRI, AAR and API in the training group (0.85, 0.81, 0.50, and 0.77 respectively) and the validation group (0.85, 0.80. 0.38 and 0.68 respectively).

Figure 4.4 ROC curves for using the APGA index, APRI AAR, and AP index in training subjects (A) and validation subjects (B) in predicting liver stiffness >8.1 kPa (severe fibrosis)

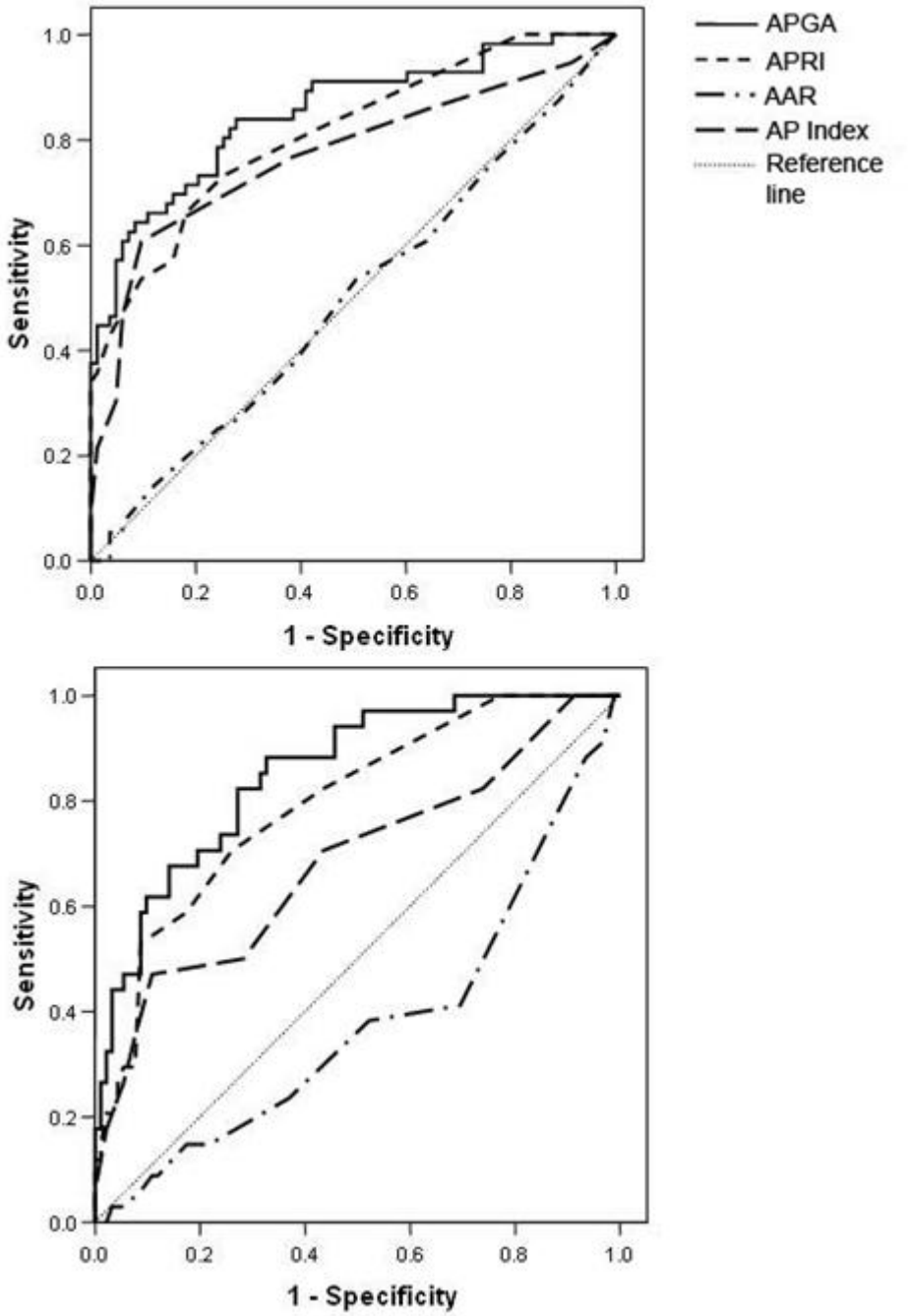

Figure 4.5 ROC curves for using the APGA index, APRI AAR, and AP index in training subjects (A) and validation subjects (B) in predicting liver stiffness >10.3 kPa (cirrhosis)

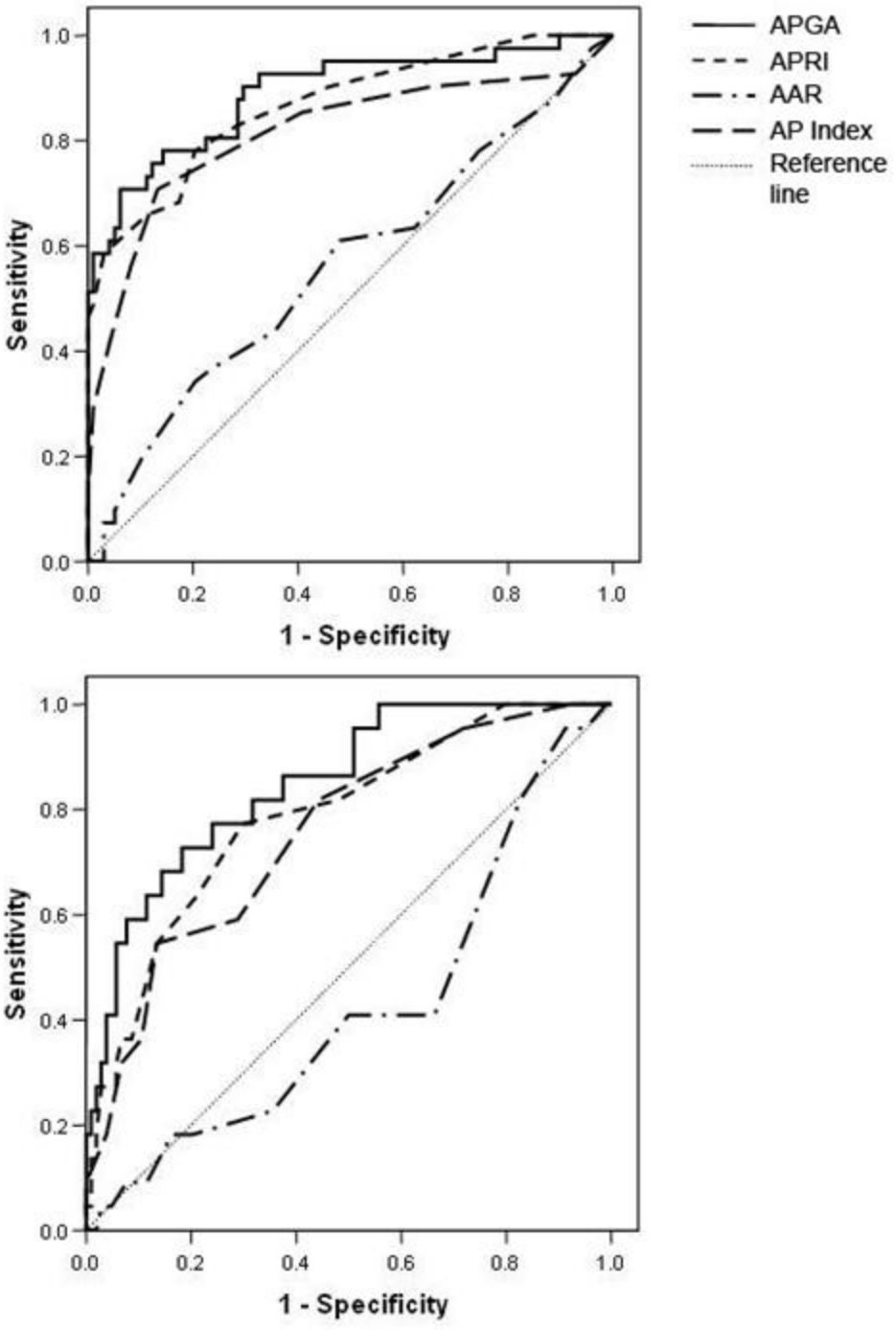

Table 4.6 AUROC using APGA, APRI, AAR, and API for fibrosis and cirrhosis in training and validation sets

	AUROC for fibrosis	**AUROC for Cirrhosis**
Training		
APGA index	0.85 (0.79-0.92)	0.89 (0.82-0.96)
APRI	0.81 (0.74-0.89)	0.87 (0.80-0.94)
AAR	0.50 (0.40-0.60)	0.56 (0.45-0.67)
API	0.77 (0.69-0.86)	0.82 (0.73-0.91)
Validation		
APGA index	0.85 (0.78-0.92)	0.85 (0.76-0.93)
APRI	0.80 (0.71-0.88)	0.79 (0.68-0.89)
AAR	0.38 (0.26-0.49)	0.43 (0.29-0.56)
API	0.68 (0.57-0.80)	0.76 (0.65-0.87)

APGA index=AST/Platelet/GGT/AFP index. APRI=AST/Platelet ratio index. AAR=AST/ALT ratio. API=age/platelet index.

Discussion

This is the first large scale study of liver stiffness measurements in Chinese CHB patients with respect to patient demographics and laboratory parameters. Previous studies have shown male sex and HBeAg-positive CHB to have more severe disease, both consistent with findings in the current study (Beasley, 1988; McMahon, Alberts, Wainwright, Bulkow, & Lanier, 1990; Yang et al., 2002). Age was also shown to directly correlate with increased liver stiffness scores. The lack of difference in the younger age groups is probably because younger patients are still in the immunotolerant phase and significant fibrosis and cirrhosis has not yet been established. However a gradient of increasing liver stiffness was noted from the age of 35 onwards, suggesting that the process of fibrosis begins mostly at around the fourth decade of life.

In the current study there was a direct correlation between serum ALT levels and liver stiffness scores. Since even those who had ALT levels in the upper limit of 'normal'(0.5-1.0 x ULN) had higher stiffness measurements compared with those who had ALT less than 0.5 x ULN, the optimal ALT levels for patients with CHB should be below 0.5 x ULN. The results are consistent with a previous study showing that CHB patients with ALT less than 0.5 x ULN have the lower complication rate than patients with ALT between 0.5-1 x ULN (Yuen et al., 2005).

Previous studies on non-invasive markers have been validated predominantly in chronic hepatitis C and not CHB patients (E. G. Giannini et al., 2006; Poynard & Bedossa, 1997; Snyder et al., 2006; Wai et al., 2003). Other studies have included special markers which are not used in

routine clinical practice, making them less useful (Imbert-Bismut et al., 2001; Poynard et al., 2004; Poynard et al., 2005). A more recent study have shown that using common biochemical markers including HBV DNA, ALP, albumin and platelet counts can reliably predict significant fibrosis in HBeAg-negative CHB patients (Mohamadnejad et al., 2006). In the current study, we have derived a model based on four readily available tests to predict liver stiffness measurements of over 8.1 kPa and 10.3 kPa in CHB patients. Studies have shown that for different underlying liver pathologies, there is a variation of optimal cut-off levels of liver stiffness values for different fibrosis stages. For chronic hepatitis C the optimal cut-off is 9.5-9.6 kPa for severe fibrosis (fibrosis stage 3 or greater). For CHB, the cut-off for severe fibrosis was lower at 8.1 kPa, and 10.3 kPa for cirrhosis. This was based on a study of 220 CHB patients who underwent both liver stiffness measurements and liver biopsy (Marcellin et al., 2005).

The four routine markers used in the current model included platelet counts, AST, GGT and AFP. The addition of extra variables including age, gender, albumin, ALT, ALP, globulin, HBV DNA and HBeAg status did not further improve the accuracy of the model. The best logarithmic model for predicting significant fibrosis was 1.44 + 0.1490log (GGT) + 0.3308log (AST) – 0.5846log (platelets) + 0.1148log (AFP+1), with an AUROC of 0.85 in both the training set and validation set. Both sensitivity and specificity were high using an optimal cut-off of 6.87 for predicting fibrosis and of 8.93 for predicting cirrhosis. By using a higher cut-off liver stiffness measurement of 12 kPa, the AUROC from our current model would improve to 0.92 with a specificity of 92% and a NPV of 96% for predicting cirrhosis. The AUROC for the current model was higher than those for the APRI, API or the AAR in both the training and validation group in predicting severe fibrosis and cirrhosis based on liver stiffness measurements. To

confirm these findings, further validation studies with liver histology is required to compare between these non-invasive models. The current model however is not a replacement for transient elastography, but rather demonstrate a potential role of these simple markers in predicting fibrosis and cirrhosis.

Although the ALT and HBV DNA levels fluctuate during the course of CHB, our final model did not include ALT or HBV DNA, as statistically neither of these added further accuracy to the model. Our model adopted AST as one of the parameters, and it has been shown that AST is more reflective of liver fibrosis and cirrhosis (Wai et al., 2003).

There are several limitations of the current study. Firstly, we have derived a predictive model based on liver stiffness measurements and not on liver histology. The definitive study would be to derive a model based on a large number of patients representing all stages of fibrosis with adequate biopsy specimens. This is increasingly difficult with the availability of safe and effective antiviral therapy bypassing the need for liver biopsy in a large proportion of CHB patients. Secondly, we have chosen an optimal liver stiffness cut-off level for Caucasian CHB patients. It would be useful to perform a validation study for Asian CHB patients correlating with liver histology.

However, the importance of the present study showed that by using the validated cut-off levels of 8.1 kPa and 10.3 kPa, the current model can predict severe fibrosis and cirrhosis with a high

AUROC by using four simple and convenient serum markers can potentially predict severe fibrosis. Finally, liver stiffness may be affected by underlying inflammatory activity, and the presence of steatosis/steatohepatitis. Although the model can accurately predict liver stiffness in Asian CHB patients, further studies are required to determine whether underlying fatty liver disease or inflammatory activity alters the liver stiffness in Asian patients with CHB.

In conclusion, liver biochemistry correlated well with liver stiffness measurements in Asian CHB patients. A model using simple serum markers can accurately predict liver stiffness, and further studies are required to assess the usefulness of these simple liver biochemical tests as non-invasive markers of fibrosis in CHB.

Chapter V

PREVALENCE OF LIVER FIBROSIS USING TRANSIENT ELASTOGRAPHY IN CHRONIC HEPATITIS B POPULATION

Introduction

In the Asian population, the majority of patients acquire hepatitis B either at birth or in the early years of life, thereby leading to a high rate of chronic infection (Lok & Lai, 1988). Up to 40% will progress to end stage liver disease including cirrhosis and HCC (Lee, 1997; Maddrey, 2000). The progression of fibrosis to cirrhosis usually occurs in a step-wise manner from mild to moderate to severe fibrosis, and finally progressing into overt cirrhosis. In Hong Kong where HBV infection is endemic (affecting approximately 7-8% of the general population), the prevalence of fibrosis is not known. This is not surprising given that for decades the only method widely available to assess liver fibrosis was by performing liver biopsy. Clearly this is not feasible for population studies and in otherwise healthy and asymptomatic individuals. However, with the availability of non-invasive methods to assess liver fibrosis, large population studies are now becoming possible to both assess the prevalence of fibrosis and its progression in the natural history of CHB.

The aims of this study were to describe the prevalence of significant fibrosis and cirrhosis in a large population of Asian CHB patients using transient elastography, to identify significant factors associated with severe fibrosis, and to compare patients who have received antiviral therapy with those patients who were treatment-naïve.

Patients and Methods

Patients

All CHB patients who attended the Hepatitis Clinic at Queen Mary Hospital during the period of January 2006 to April 2007 were invited to undergo liver stiffness measurement with transient elastography. CHB infection was defined as having HBsAg positive for 6 months or more. Those with HCV co-infection, autoimmune liver disease, Wilson's disease, PBC or significant alcohol intake (>20g/day) were excluded. Patient demographics and laboratory parameters were recorded at the time of liver stiffness measurement, including age, sex, bilirubin, ALT, albumin, AFP, HBsAg, HBeAg, anti-HBe, and serum HBV DNA levels. HBV DNA was measured using the COBAS Taqman® HBV DNA Assay [Roche Diagnostics, Branchburg, NJ] with a lower limit of detection of 60 copies/ml. The HBV DNA level of 60 copies/ml was used for patients with undetectable levels for statistical calculations. This study was approved by the Institutional Review Board of the University of Hong Kong.

Measurement of liver stiffness

The method of measuring liver stiffness has been described in previous chapters. Liver stiffness score of >7.0 kPa, >8.1 kPa, and >10.3 kPa were used as cut-offs for the presence of at least significant fibrosis, severe fibrosis, and cirrhosis respectively (Marcellin et al., 2005).

Statistics

All statistical analyses were performed using the SPSS version 14.0 (SPSS Inc, Chicago, IL). Chi-squared test was used for categorical variables, and Fischer's exact test when appropriate. Continuous variables with skewed distribution were analyzed using Mann-Whitney test. Multivariate analysis was performed using binary logistic regression on variables significant on univariate analysis. A 2-sided p-value of <0.05 was considered statistically significant.

Results

A total of 1394 CHB patients underwent transient elastography between the period January 2006 and April 2007. Of these, 79 patients were excluded from the analysis because of failure to obtain a valid stiffness score in 24 (2%) patients, and 55 (4%) patients had suboptimal scans as defined by valid results of less than 50%. Patients who had failed scans were either due to obesity, narrow intercostal space, or overlying adipose tissue. Of the remaining 1315 patients, 851 patients did not receive any antiviral therapy (treatment-naïve group) and 364 patients had prior or current antiviral therapy (treatment group). Of the treated patients, 280 patients had received either lamivudine or adefovir, 63 patients received interferon-based therapy, and 21 received both oral nucleoside analogs and interferon therapy. The prevalence of severe fibrosis (>8.1 kPa) and its associated factors were reported for treatment-naïve patients. The treatment group was included as a comparison group with treatment-naïve patients.

In the treatment-naïve group, 605 were male (64%) and 346 were females (36%). The median age was 43 years (range, 17 to 88 years). The HBeAg was positive in 226 (24%) and 725 (76%) patients were HBeAg-negative. The median liver stiffness was 6.7 kPa (range, 1.5-75.0) with a median IQR of 1.2 (0-24.9). The demographic and liver biochemistry results are summarized in table 5.1.

Table 5.1 Demographic and laboratory results of patients

Parameter	Value
Total patients undergoing transient elastography	1394
Failed scan	24 (2%)
Suboptimal scan	55 (4%)
Valid scans	1315
Treated patients	364
Treatment-naïve patients	951
Liver stiffness (kPa)	6.7 (1.5-75.0)
Interquartile range	1.2 (0-24.9)
Success rate	100% (50-100%)
Gender	
Male	605 (64%)
Female	346 (36%)
Age (median)	43 years (17-88)
HBeAg	
Positive	226 (24%)
Negative	725 (76%)
Liver biochemistry	
Bilirubin (umol/L)	11 (2-172)

ALT (U/L)	35 (8-962)
AST (U/L)	26 (6-282)
ALP (U/L)	68 (23-300)
GGT (U/L)	24 (8-370)
Albumin (g/L)	44 (25-51)
HBV DNA (copies/ml)	229,890 (60-640,000,000)

* All continuous variables are expressed as median values

The prevalence of significant fibrosis (>7.0 kPa), severe fibrosis (>8.1 kPa) and cirrhosis (>10.3 kPa) is summarized in Table 5.2. Overall, 319 (34%) had liver stiffness >8.1 kPa with a higher proportion of male patients compared with female patients (39% vs 24% respectively, $p<0.001$). There was no significant difference in the proportion of patients with liver stiffness >8.1 kPa between HBeAg-positive and HBeAg negative patients (36% vs 33% respectively, $p=0.40$). Similar trends were seen for patients with liver stiffness >7.0 kPa and >10.3 kPa.

Table 5.2 Prevalence of at least significant fibrosis, severe fibrosis, and cirrhosis

Patients	Significant Fibrosis (>7.0 kPa)	Severe Fibrosis (>8.0 kPa)	Cirrhosis (>10.3 kPa)
Total	429 (45%)	319 (34%)	213 (22%)
Males	311 (51%)	236 (39%)	165 (27%)
Females	118 (34%)	83 (24%)	48 (14%)
	p<0.001	p<0.001	p<0.001
HBeAg-positive	105 (45%)	81 (36%)	57 (25%)
HBeAg-negative	324 (47%)	238 (33%)	156 (22%)
	p=0.76	p=0.40	p=0.29

The prevalence of patients with liver stiffness >8.1 kPa stratified into different age groups and according to gender is shown in table 5.3. There was increasing prevalence of liver stiffness >8.1 kPa seen with age: from 20% in those under age 25 years to 81% in those over the age of 65 years. Below the age of 35 there was no difference in prevalence of patients with liver stiffness >8.1 kPa between male and female patients. Between the age of 36 to 45 and 46 to 55 there was significantly more male patients with liver stiffness >8.1 kPa compared with female patients (38% vs 20% and 45% vs 28% respectively, both p<0.001). The difference between males and

females became less significant in patients aged 56 to 65 years (65% male vs 43% females, p=0.06). No significant difference was observed after the age of 65 years.

The prevalence of those with liver stiffness >8.1 kPa in HBeAg-positive and HBeAg-negative patients stratified by different age groups is shown in table 5.4. In patients who were ≤45 years old, there was no significant difference in prevalence between HBeAg-positive and HBeAg-negative patients. In patients over the age of 45 years, those who were HBeAg-positive had higher rates of liver stiffness >8.1 kPa compared with HBeAg-negative patients (58% vs 43%, p=0.03)

Patients with higher HBV DNA levels also had significantly higher proportion with liver stiffness >8.1 kPa as shown in table 5.5. The lack of significant difference between patients with HBV DNA below 3 log copies/ml compared with patients with HBV DNA above this level is probably due to the relatively small number of patients with HBV DNA <3 log copies/ml (n=12).

There was also increasing prevalence of patients with liver stiffness >8.1 kPa with increasing ALT levels: in patients with ALT less than 0.5 x upper limit of normal (ULN) and 0.5-1 x ULN (11% vs 30% respectively, p<0.01) and 0.5-1 x ULN and 1-2 x ULN (30% vs 48% respectively, p<0.01). There was borderline significance in those with 1-2 x ULN and 2-5 x ULN (48% and 59% respectively, p=0.06) and no significant differences between those with ALT 2-5 x ULN and >5 x ULN (table 5.6).

Table 5.3 Prevalence of liver stiffness >8.1 kPa in males and females stratified by age groups

Age groups (years)	Total	Male	Female	p-value (male vs female)
≤ 25 (n=60)	12 (20%)	9 (22%)	3 (16%)	0.43
26-35 (n=195)	36 (18%)	22 (19%)	14 (17%)	0.44
36-45 (n=318)	98 (31%)	72 (38%)	26 (20%)	<0.01
46-55 (n=282)	112 (40%)	87 (45%)	25 (28%)	<0.01
56-65 (n=75)	44 (59%)	34 (65%)	10 (43%)	0.06
>65 (n=21)	17 (81%)	12 (75%)	5 (100%)	0.30

Table 5.4 Prevalence of liver stiffness >8.1 kPa in HBeAg-positive and HBeAg-negative patients stratified by age groups

Age groups (years)	HBeAg positive	HBeAg negative	p-value
≤ 25 (n=60)	6 (24%)	6 (17%)	0.51
26-35 (n=195)	16 (26%)	20 (15%)	0.07
36-45 (n=318)	21 (29%)	77 (31%)	0.67
>45 ((n=378)	38 (58%)	135 (43%)	0.03

Table 5.5. Prevalence of liver stiffness >8.1 kPa in patients according the HBV DNA levels

HBV DNA levels Log (copies/ml)	Patients	P value
< 3 log	12 (30%)	0.25
≥ 3 log	147 (38%)	
< 4 log	30 (27%)	<0.01
≥ 4 log	134 (41%)	
< 5 log	50 (27%)	<0.01
≥ 5 log	114 (45%)	
< 6 log	81 (32%)	0.01
≥ 6 log	83 (45%)	
< 7 log	106 (34%)	0.03
≥ 7 log	58 (45%)	

Table 5.6. Prevalence of liver stiffness >8.1 kPa in patients stratified by different ALT groups

ALT levels	Number	P value (between groups)
0.5 x ULN	23 (11%)	
		< 0.01
0.5–1 x ULN	114 (30%)	
		<0.01
1–2 x ULN	114 (48%)	
		0.06
2–5 x ULN	51 (59%)	
		0.72
>5 x ULN	9 (64%)	

In total there were 486 patients who had completely normal liver biochemistry with normal ALT levels, bilirubin ≤20 umol/L and albumin ≥40g/L. Of these, 89 (18%) had liver stiffness >8.1 kPa. For those patients over the age of 45 years and with normal liver enzymes (n=211), 34% had liver stiffness >8.1 kPa.

Using multivariate analysis by regression to determine factors associated with liver stiffness >8.1 kPa, gender, age and ALT were significant factors (both $p<0.001$), whereas HBV DNA was not significant ($p=0.25$).

Non-treatment versus Treatment Group

The 951 treatment-naïve patients were compared to 364 patients who had received antiviral therapy either prior to or at the time of liver stiffness measurement. The patients in the treated groups were slightly older than the treatment-naïve group. There was no significant difference in measurements ALT, albumin, liver stiffness and prevalence of severe fibrosis between the two groups. The results are summarized in table 5.7. The patients who received antiviral therapy were divided into those with treatment success as defined by a normal ALT at the time of last follow-up. Of the 364 treated patients, 249 (68%) had successful treatment. Patients with successful treatment had a lower ALT, lower liver stiffness score and lower prevalence of cirrhosis compared with those patients who were treatment-naïve [25 vs 35 U/L ($p<0.001$), 6.2 vs 6.7 kPa ($p=0.031$) and 14% vs 22% ($p=0.008$) respectively].

Table 5.7. Comparison between treated (overall) patients versus non-treated patients, and in those with successful treatment versus non-treat patients

Parameters	Treated	No treatment	P value
Age (years)	45 (18-86)	43 (17-88)	0.016
ALT (U/l)	32 (9-1109)	35 (8-962)	0.406
Albumin (g/l)	43 (22-51)	44 (25-51)	0.539
Liver stiffness (kPa)	6.8 (1.5-67.8)	6.7 (1.5-75.0)	0.603
Liver stiffness >8.1 kPa	136 (37%)	322 (34%)	0.218
	Treated (Success)	No treatment	
Age (years)	44 (18-86)	43 (17-88)	0.264
ALT (U/l)	25 (9-52)	35 (8-962)	<0.001
Albumin (g/l)	44 (22-51)	44 (25-51)	0.058
Liver stiffness (kPa)	6.2 (2.8-47.3)	6.7 (1.5-75.0)	0.031
Liver stiffness >7.0 kPa	95 (38%)	426 (48%)	0.060
Liver stiffness >8.1 kPa	68 (27%)	322 (34%)	0.061
Liver stiffness >10.3 kPa	36 (14%)	210 (22%)	0.008

Discussion

In the current study we have defined the population prevalence of probable severe fibrosis and cirrhosis in Chinese CHB patients by liver stiffness measurement using transient elastography. A total of 79 (6%) patients were excluded from the study because of failed scans or suboptimal scans due to increased body mass index or overlying adipose tissue. This is consistent with a previous large study showing a failure rate of 5% with increased body mass index being the only significant factor (Foucher, Castera et al., 2006).

Previous studies have shown male sex and HBeAg-positive CHB to have more severe disease (Beasley, 1988; McMahon et al., 1990; Yang et al., 2002; Yuen et al., 2005). In the present study, we have further identified that both gender and HBeAg status differences were dependent on age. There was no significant difference between males and females below age 35 years. However from age 36-65 years, significantly higher proportion of males had liver stiffness >8.1 kPa compared with females. This difference was no longer seen in patients after the age of 55 years. The lesser degree of fibrosis in females was restricted to the child bearing age. This raises the possibility of estrogen having a protective effect on the development of liver fibrosis, an effect also suggested by previous studies (Codes et al., 2007; Di Martino et al., 2004). This may be analogous to the effect estrogen has on atherosclerosis and cardiovascular disease with the protective effect being reduced after menopause (Klouche, 2006; Rosano, Vitale, Marazzi, & Volterrani, 2007). However, further studies are required to confirm the potential effect of estrogen on the liver.

The difference in prevalence of those with liver stiffness >8.1 kPa in HBeAg-positive and HBeAg-negative patients was seen only after the age of 45 years. Persistent HBeAg positivity probably means a prolonged immune-clearance phase with prolonged immune damage to the liver, leading to more fibrosis. In addition, in patients over the age of 45 years with normal liver enzymes, 34% had liver stiffness >8.1 kPa. Recent practice guidelines recommend that patients who remain HBeAg-positive after the age of 40 with HBV DNA >20,000 IU/ml should be considered for liver biopsy to assess for significant fibrosis (Keeffe et al., 2006; Lok & McMahon, 2007). However, even in HBeAg-positive patients under the age of 25 years, 24% had liver stiffness >8.1 kPa, suggesting that even in the relatively early stage of immune clearance, there may already be significant underlying disease.

The present study shows a higher viral load was associated with higher liver stiffness (>8.1 kPa). However after multivariate analysis, HBV DNA was not a significant factor, whereas age, ALT and gender remained significant factors. This may be related to the fact that the HBV DNA was determined at a single time-point and not longitudinally. The damage to the liver might already have occurred by the time the HBV DNA level was determined in our study. It has been shown that HBV DNA levels change over time (C. J. Chen et al., 2006; Hui et al., 2007).

There is increasing evidence to suggest that the upper threshold defining normal ALT is likely to be lower than the current accepted values. Our current study shows that patients with ALT levels <0.5 x ULN have significantly lower rate of liver stiffness >8.1 kPa compared to patients with ALT 0.5-1 x ULN, 1-2x ULN, and 2-5 x ULN (11% vs 30% vs 48% vs 59% respectively). A

recent large population study of CHB patients also showed that patients with ALT <0.5 x ULN had the best long-term prognosis (Yuen et al., 2005). In two large population studies of subjects with no known liver diseases and normal ALT, there were positive correlations with ALT concentration and liver-related mortality. Subjects with ALT levels in the upper ranges of normal also had significantly higher liver-related mortality compared with those with ALT levels below 0.5 x ULN (Kariv et al., 2006; Kim et al., 2004). Subsequent to these studies, the accepted ULN for ALT levels have been revised to a lower level of 30 and 19 U/L for males and females respectively (Lok & McMahon, 2009). In patients with completely normal liver biochemistry including normal ALT levels, albumin and bilirubin, the prevalence of patients with liver stiffness >8.1 kPa was still 18%. These patients with significant underlying disease should be considered for antiviral therapy or at least closer disease surveillance.

Although there was no overall difference in liver stiffness between patients who had received or were receiving antiviral therapy compared to treatment-naïve patients, successful antiviral therapy may prevent severe fibrosis and cirrhosis with significantly lower liver stiffness and prevalence of cirrhosis. The lack of difference between the overall population receiving antiviral therapy and the treatment-naïve patients may be due to selection of patients with more severe baseline disease for treatment. This might have negated any detectable differences when compared with treatment-naïve patients.

There are several limitations of the current study. Firstly, liver stiffness measurements were performed on unselected CHB patients seen at our Hepatitis Clinic, with referrals from both

primary care physicians and community centers to our tertiary center. However referral bias is minimal since the majority of the referrals were from transfusion services and family planning clinics. Nearly all the patients were asymptomatic. The referrals were also accepted regardless of the ALT levels. The very low number of patients with undetectable HBV DNA however would suggest that some degree of referral bias will be present. Secondly we do not have histological confirmation of severe fibrosis, and this is not feasible in a large population study of predominantly asymptomatic patients. We have chosen an optimal liver stiffness cut-off level for Caucasian CHB patients. It will be useful to perform a validation study for Asian CHB patients correlating with liver histology, although theoretically the liver stiffness should not be different.

In conclusion, this study shows that the overall prevalence of liver stiffness >8.1 kPa and >10.3 kPa suggestive of severe fibrosis and cirrhosis respectively in Chinese CHB patients was significant at 34%. The higher proportion of patients with liver stiffness >8.1 kPa observed in males and positive HBeAg status was an age-specific phenomenon. In addition, the risk of having liver stiffness >8.1 kPa occurred along a gradient of ALT levels, with the lowest risk seen in patients with ALT below half the ULN. Finally, successful treatment with antiviral therapy might lower risk of severe fibrosis and cirrhosis.

Chapter VI

EFFECT OF SEVERE HEPATITIS B FLARES ON LIVER STIFFNESS MEASUREMENTS

Introduction

Although liver stiffness measurement using transient elastography has been shown to correlate well with the severity of liver fibrosis, there may be other potential factors which may affect liver stiffness. For instance, the amount of inflammation or the degree of hepatitis in patients with CHB may have an effect on liver stiffness. The marked increase in lymphocytic and other cellular infiltration may alter the stiffness of the liver.

In hepatitis B, severe hepatitis can occur at the time of HBV infection (acute hepatitis B), or as a flare up of CHB infection. We conducted a prospective study to determine the effect of severe flare of hepatitis B on liver stiffness measurements over a 12 month period.

Patients and Methods

This was a prospective study. All CHB patients, defined by HBsAg positive for more than six months, with hepatitis B flare of ALT greater than 10x ULN, and admitted to Queen Mary Hospital, Hong Kong, between the period May 2006 and August 2007, were included. All patients had HBV DNA levels $>10^5$ copies/ml. Acute hepatitis A and E were excluded by serological testing. Drug-induced hepatitis was excluded by careful history taking. None of the patients included had underlying significant alcohol intake as defined by a daily intake of >20g/day. All patients gave informed consent prior to the testing for liver stiffness measurements. Patient demographics and laboratory parameters were recorded at the time of liver stiffness measurement, including age, sex, liver biochemistry, HBsAg, HBeAg, anti-HBe, and serum HBV DNA levels. HBV DNA was measured using the COBAS Taqman® HBV DNA Assay [Roche Diagnostics, Branchburg, NJ], with a lower detection limit of 60 copies/mL.

Liver stiffness measurement

Only patients with at least 10 valid measurements and a success rate of over 60% were included. Liver stiffness scores were expressed in units of kilopascals (kPa). This was performed in all patients at the time of admission, 3-6 months, and 12 months after the time of flare. A subgroup also had liver stiffness determined at 4 weeks after flare. We adopted the value of <6.0 kPa as normal liver stiffness, as defined by a previous study (Roulot et al., 2008).

Liver histology

Five patients underwent liver biopsy during their hepatitis B flare. Three patients were admitted for hepatitis B flare outside the recruitment period (with the same inclusion and exclusion criteria as listed). The necro-inflammatory scores and degree of fibrosis were assessed according to the modified HAI grading and staging system (Ishak et al., 1995; Knodell et al., 1981).

Statistical analysis

All statistical analyses were performed using the SPSS version 14.0 (SPSS Inc, Chicago, IL). Chi-squared test was used for categorical variables, and Fisher's exact test when appropriate. Continuous variables with skewed distribution were analyzed using Mann-Whitney test. Paired related data were analyzed using the Wilcoxon paired test. Correlation between liver stiffness and liver biochemistry was performed using Spearman's bivariate correlation. A p-value of <0.05 was considered statistically significant.

Results

A total of 38 patients were recruited during the study period. Five patients were excluded due to inadequate follow-up, and a further 4 patients was excluded due to a success rate of <60% in any one of their scans at the 3 different time points. Twenty-nine patients were included in the final analysis. One patient had documented evidence of fatty liver disease on ultrasonography. The patient's demographic, baseline laboratory data, and liver stiffness measurements are summarized in table 6.1.

Eleven (38%) patients were asymptomatic with abnormal liver enzymes discovered on regular blood surveillance. Eight (28%) patients presented with jaundice, and 10 (35%) patients presented with non-specific viral symptoms without jaundice. None of the patients had normal liver stiffness (<6.0 kPa) at the time of hepatitis flare; the median liver stiffness was 16.8 kPa (range, 6.9-47.2). Twenty-four (83%) patients were treated with oral nucleoside/nucleotide analogues.

Table 6.1 Patient demographics and baseline laboratory values

Parameters	Value
Total patients (n)	38
Inadequate follow-up	5
Invalid scans (success<60%)	4
Final analysis	29
Sex (Male)	23 (79%)
Age (years)	44 (20-69)
HBeAg -positive	16 (55%)
Bilirubin (umol/L)	34 (10-469)
Alkaline phosphatase (U/L)	114 (56-220)
GGT (U/L)	159 (48-540)
AST (U/L)	524 (208-1678)
ALT (U/L)	1464 (594-2476)
Albumin (g/L)	39 (27-46)
Prothrombin time (s)	12.8 (11.1-24.6)
Platelets (10^9/L)	185 (78-313)
HBV DNA (copies/mL)	100,000,000 (646020-640,000,000)
Liver stiffness measurement (kPa)	16.8 (6.9-47.2)
Interquartile ratio**	17% (4-47%)

<10 kPa	7 (24%)
10-20 kPa	10 (35%)
>20 kPa	12 (41%)

* Continuous variables expressed as median values.

** Interquartile ratio = Interquartile range/liver stiffness. 86% of patients had ratio ≤30%

At 3-6 months follow-up

At 3-6 months, there was a significant decline in liver stiffness from a median baseline level of 16.8 kPa (range, 6.9-47.2) to 7.9 kPa (range, 4.6-20.4); ($p<0.001$). Eight (28%) patients had normalized their liver stiffness results, as defined by a liver stiffness measurement of <6 kPa, whereas no patients at baseline had normal liver stiffness measurement. Patients who normalized their liver stiffness at 3-6 months had significantly lower liver stiffness at baseline compared to patients with abnormal liver stiffness at 3-6 months (9.0 vs 20.4 kPa respectively, $p=0.021$). There was no significant difference in the age, and baseline levels of bilirubin, ALT, platelets, albumin, and prothrombin time, between patients with or without normal liver stiffness at 3-6 months. Twenty-six (90%) had normalized their ALT, with the remaining 3 patients only having minimally elevated ALT of less than twice the upper limit of normal. The results are summarized in table 6.2.

At 12 months follow-up

At 12 months, the median liver stiffness was 6.9 kPa (range, 3.3-23.6), which was significantly lower when compared to liver stiffness at 3-6 months after flare ($p=0.039$). Ten patients (34%) had normalized their liver stiffness after 1 year. All patients had normalized their ALT at 12 months after initial flare. The results are summarized in table 6.2. Only one patient had HBV DNA level $>10^5$ copies/mL (101,850 copies/mL), and 59% had undetectable HBV DNA.

Table 6.2 Comparison of liver stiffness and liver biochemistry at baseline, 3-6 months, and at 12 months

Parameters	Baseline	3-6 months	12 months
Total bilirubin (umol/L)	34 (10-469)	12 (4-39)	12 (2-46)
ALT (U/L)	1464 (594-2476)	30 (11-66)	21 (13-46)
Albumin (g/L)	39 (27-46)	44 (36-52)	45 (38-49)
Liver stiffness (kPa)	16.8 (6.9-47.2)[†]	7.9 (4.6-20.4) [†‡]	6.9 (3.3-23.6) [‡]

* Continuous variables expressed as median values.

[†] $p<0.001$ comparing liver stiffness value between baseline and 3-6 months

[‡] $p=0.039$ comparing liver stiffness value between 3-6 months and 12 months

Five (17%) patients had the diagnosis of liver cirrhosis at 12 months, defined by a liver stiffness value of >10.3 kPa. Baseline factors associated with the diagnosis of cirrhosis at 12 months included a higher ALP level (164 vs 107 U/L respectively, $p=0.027$) and liver stiffness (40.3 vs 14.0 kPa respectively, $p=0.016$), and lower albumin level (35 g/L vs 40 g/L respectively, $p<0.001$), compared to those patients that did not have cirrhosis. The baseline age, bilirubin, AST, ALT, prothrombin time, platelets, and HBV DNA levels were not significantly different between these two groups.

The median liver stiffness measurements at baseline, 3-6 months, and at 12 months are summarized in figure 6.1. The serial liver stiffness measurements of every individual patient are shown in figure 6.2. The changes in liver stiffness between different time points are shown in figure 6.3.

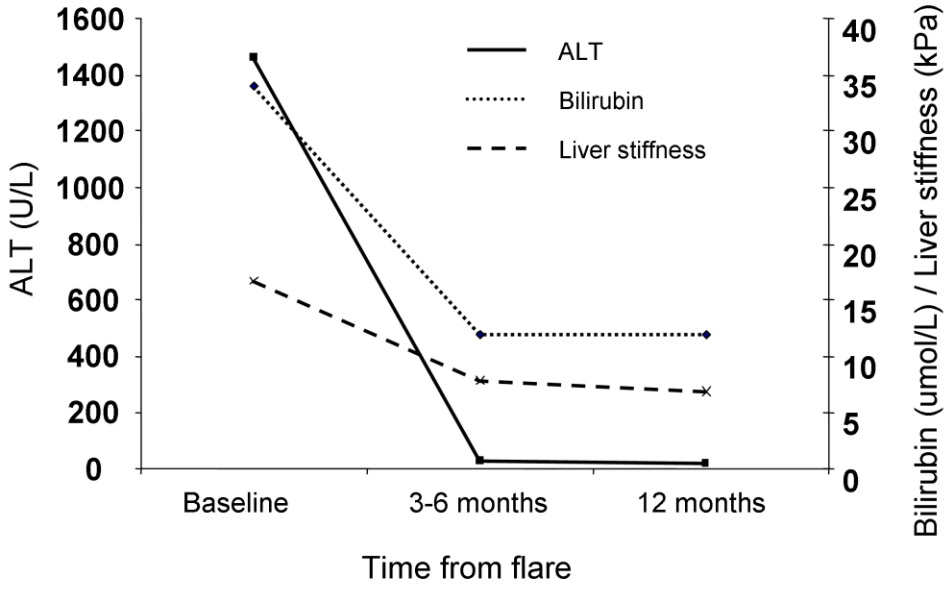

Figure 6.1 Median levels of bilirubin, ALT, and liver stiffness at baseline, 3-6 months and 12 months

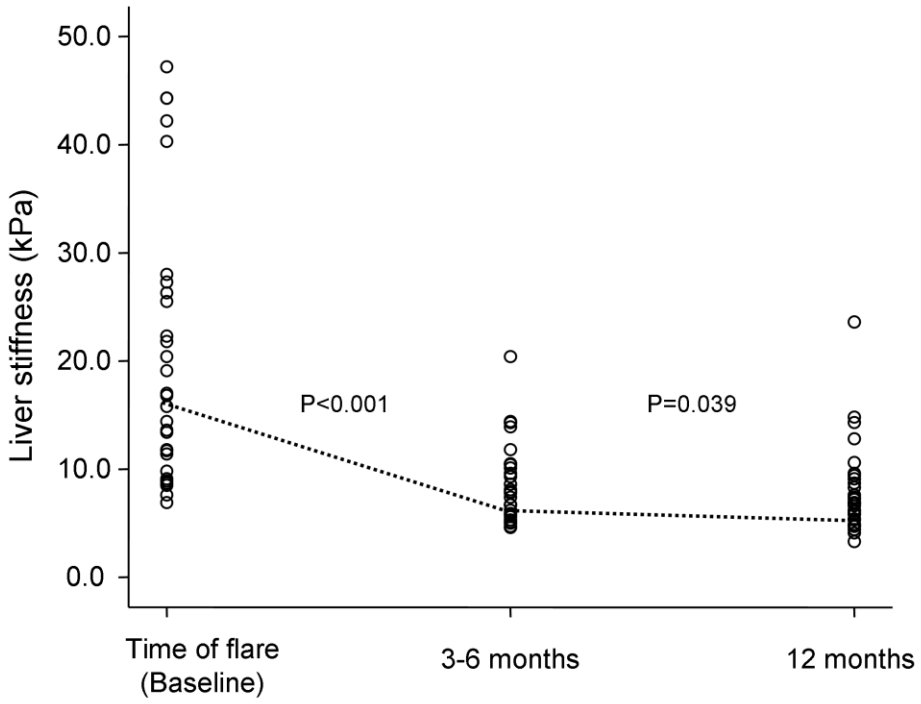

Figure 6.2 Distribution of liver stiffness of all patients at baseline, 3-6 months, and at 12 months.

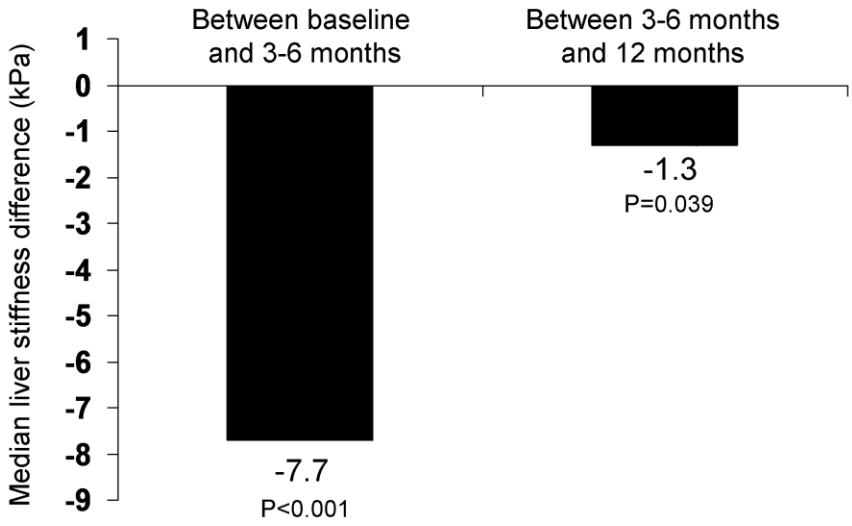

Figure 6.3 Median reduction in liver stiffness between baseline and 3-6 months, and between 3-6 months and 12 months

Further follow-up beyond 12 months

Of the 29 patients, 25 was available for repeat liver stiffness measurement at the time of last follow-up, beyond the 12 months study period, with a median time of 34 months from the time of flares. There was a further decline of liver stiffness of 1.0 kPa (p=0.02) between the last follow-up time and at 12 months. None of these 25 patients had liver stiffness measurements of >10.3 kPa.

Early decline in liver stiffness

A subgroup of 15 patients (52%) was available at week 4 for early follow-up and had liver stiffness measurements performed at this visit. There was a significant decline at 4 weeks compared to baseline (8.8 vs 11.8 kPa respectively, p=0.005). At 4 weeks, the median ALT was 103 U/L (range, 14-437), compared to the baseline level of 1532 U/L (range, 612-2476). The results are shown in figure 6.4. Of those patients that had early decline in liver stiffness, there was no difference in the HBV DNA levels at 12 months when compared to those patients that did not have an early decline (144 vs 60 copies/mL, p=0.17). Therefore, early decline in liver stiffness was not predictive of viral suppression after 1 year.

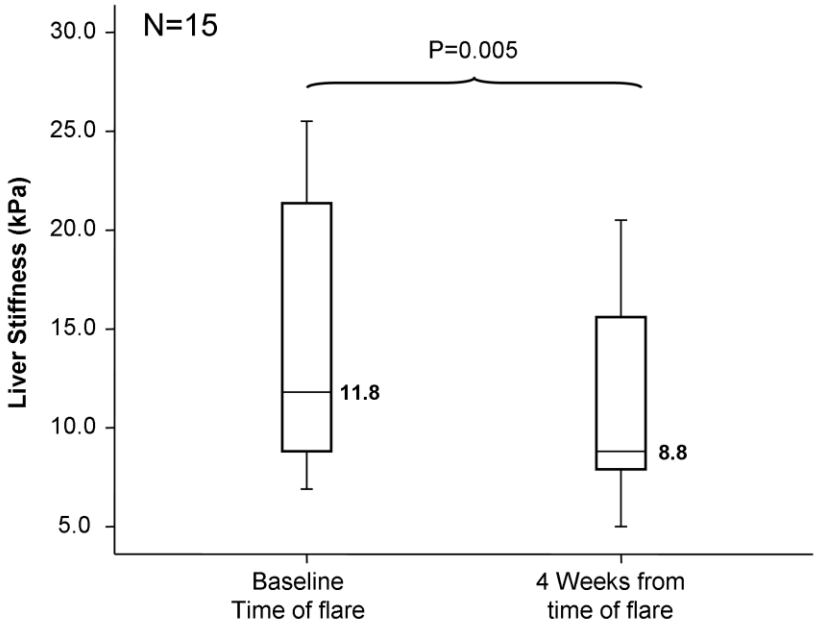

Figure 6.4 Median liver stiffness at baseline and at 4 weeks (subgroup analysis of 15 patients)

Correlation of baseline parameters and liver stiffness

The baseline parameters and its correlation are summarized in table 6.3. The age, HBeAg status, levels of ALP, GGT, AST and HBV DNA, did not significantly correlate with liver stiffness. Baseline factors which correlated with liver stiffness measurements included bilirubin, ALT, albumin, prothrombin time, and platelet levels ($p=0.010$, $p=0.047$, $p=0.010$, $p<0.001$ and $p=0.004$ respectively). The correlation co-efficient between baseline parameters and liver stiffness at 3-6 months for bilirubin, ALT, albumin, prothrombin time, and platelet levels were 0.346 ($p=0.066$), 0.220 ($p=0.252$), -0.458 ($p=0.013$), 0.474 ($p=0.011$), and -0.227 ($p=0.245$) respectively. The correlation co-efficient between baseline parameters and liver stiffness at 12 months for bilirubin, ALT, albumin, prothrombin time, and platelet levels were 0.324 ($p=0.086$), 0.090 ($p=0.642$), -0.499 ($p=0.006$), 0.321 ($p=0.096$), and -0.110 ($p=0.577$) respectively. There were also significant correlations between the liver stiffness measurement and ALT levels at 3-6 months (correlation coefficient=0.449, $p=0.014$) and at 12 months (correlation coefficient=0.458, $p=0.016$).

Table 6.3 Correlation of baseline parameters with liver stiffness

Parameters	Correlation coefficient	P value
Age	-0.221	0.249
HBeAg status	0.310	0.102
Bilirubin	0.470	0.010
ALP	0.137	0.478
GGT	-0.335	0.110
AST	0.353	0.060
ALT	0.371	0.047
Albumin	-0.471	0.010
Prothrombin time	0.687	<0.001
Platelet	-0.532	0.004
HBV DNA	0.310	0.102

* Spearman's correlation coefficient

There was no significant difference in baseline liver stiffness between HBeAg-positive and HBeAg-negative patients (16.8 vs 16.4 kPa respectively, p=0.942). After 6 months, there was also no significant difference in liver stiffness between HBeAg-positive and HBeAg-negative patients (7.3 vs 9.6 kPa respectively, p=0.178). There was also no significant correlation with baseline HBV DNA with liver stiffness at baseline and at 6 months after flares (p=0.102 and p=0.272 respectively).

ALT flare and liver stiffness

The levels of ALT were stratified into groups above 1000, 1500, and above 2000 U/L. There was no difference in liver stiffness between patients with ALT>1000 U/L and in those with ALT<1000 U/L (20.4 kPa vs 16.3 kPa respectively, p=0.40), and in patients with ALT >1500 U/L compared with ALT <1500 U/L (23.9 kPa vs 15.8 kPa respectively, p=0.201). There was a significant difference in patients with ALT>2000 U/L compared with those with ALT <2000 U/L (34.3 kPa vs 14.4 kPa respectively, p=0.022).

Antiviral therapy

Twenty-four (83%) patients received nucleoside/nucleotide analogues with lamivudine (4 patients), adefovir (2 patients), or entecavir (18 patients). After 3 months, there was no significant difference in median liver stiffness reduction in patients who received antiviral therapy and those that did not (7.2 kPa vs 12.4 kPa respectively, p=0.845).

Liver histology during flare

Five patients underwent liver biopsy during their inpatient admission at the time of hepatitis B flare. Two were from this study population, and three additional patients were admitted outside the recruitment time. The results are summarized in table 6.4. The liver stiffness score was much higher than the expected value for the stage of fibrosis observed on histology.

Table 6.4 Liver stiffness, histological activity and stage of fibrosis during severe flare

Patient	Sex	Age	ALT	Liver Stiffness (kPa)	Necroinflammatory Score (max 18)	Fibrosis Score (max 6)
1	M	28	629	14.3	4	1
2	M	48	759	6.9	3	2
3	M	21	899	8.8	6	1
4	M	46	1184	20.4	8	3
5	M	24	673	34.3	5	1

* Necroinflammatory and fibrosis score according to the modified hepatic activity index grading and staging system

Discussion

Most of the initial validation studies on liver stiffness measurement using transient elastography have been performed on patients with chronic hepatitis C, with less available data on CHB. Even less data is available with regards to liver stiffness measurement during the time of hepatitis flare. A recent study performed on 18 patients with acute viral hepatitis [hepatitis A (n=7), B (n=8), and C (n=3)] has shown a significant correlation between liver stiffness and serum aminotransferase levels, concluding that liver stiffness measurement is not reliable in the context of acute hepatitis (Arena et al., 2008). In that particular study, the timing of liver stiffness measurements was performed with regard to the defined ALT levels after an episode of flare, namely at the time when ALT halved, and finally at the time when ALT was reduced to <2 x ULN. In another recent study performed by Sagir et al. of 20 patients with hepatitis flares [hepatitis A (n=1), hepatitis B (n=8), toxic (n=8), and autoimmune hepatitis (n=3)], liver stiffness was found to be elevated in the absence of severe fibrosis or cirrhosis during the time of flare (Sagir, Erhardt, Schmitt, & Haussinger, 2008). The correlation between ALT levels and the liver stiffness measurement was done by pooling the measurements from 20 patients. The longitudinal data on liver stiffness were available in a limited number of patients. In contrast to these two recent studies, the current study looks specifically at those patients with acute severe flare of hepatitis B, followed up for 1 year, with liver stiffness measurement performed at specific time points, namely, at the time of the severe flares, 1, 3 – 6, and 12 months after these flares.

We identified several significant factors which correlated with liver stiffness during an acute flare, including higher bilirubin, ALT, and prothrombin time, and lower albumin and platelet

levels. The baseline ALT and platelet levels, however was not shown to be correlated with liver stiffness at 3-6 months or at 12 months. By contrast, the baseline albumin level was shown to be inversely correlated to liver stiffness at baseline, after 3-6 months, and at 12 months. There was a trend for correlation with baseline bilirubin level and prothrombin time with liver stiffness at 12 months. This suggests that baseline parameters such as bilirubin, prothrombin time, and albumin may indicate underlying pre-existing damage to the liver already and is more predictive of long term outcome rather than the severity of the ALT rise at the time of hepatitis flare. By stratifying the ALT levels, a significantly higher liver stiffness measurement was shown only for patients with ALT higher than 2000 U/L.

Most of the decline in liver stiffness after a severe flare occurs within the first 6 months, with only further minimal reduction from 6 months to 12 months. These results suggest that after an acute hepatitis B flare, liver stiffness returns close to its background level by 3-6 months, with further minimal decline over the next 6 months. This is in keeping with a larger population study of 1,315 patients of CHB patients, whereby the median liver stiffness was found to be 6.7 kPa, which is comparable to the value of 6.9 kPa at 12 months after flare in the present study population (Fung, Lai, But et al., 2008). Although the decline in liver stiffness in our patients may be partly related to reversal of fibrosis with antiviral therapy, it is likely that the major cause of the decline was due to decrease in inflammatory activities. Liver stiffness measurements should be performed at least 6 months after resolution of severe flares (e.g. ALT <2 x ULN). If the results remain unexpectedly high, we would recommend repeating another scan in a further 6 months time, as we have shown that there is a further significant decline in liver stiffness between 3-6 months and 12 months. By 12 months, if the liver stiffness is still elevated, this is

likely to reflect underlying degree of fibrosis, and any further subsequent decline in liver stiffness thereafter is likely a reduction in fibrosis.

Whether this can be applied to other causes of severe acute flares of hepatitis, such as acute alcoholic hepatitis, autoimmune flares, acute presentation of Wilson's disease, drug-related hepatitis, or other viral hepatitis, remains to be determined. The two recent studies, having shown that liver stiffness is elevated during acute hepatitis flares from other causes, including hepatitis A, hepatitis C, toxic hepatitis, and autoimmune hepatitis, will provide strong evidence for this (Arena et al., 2008; Sagir et al., 2008). Of note, an increase liver stiffness measurement after an episode of severe flare was not predictive of the presence of underlying advanced fibrosis or cirrhosis, as all patients had lowered their liver stiffness at the time of last follow-up.

Although we have shown that liver stiffness is significantly higher during the time of hepatitis flare, the nature of this elevated liver stiffness remains to be determined. Whether the higher measurements reflect truly the increase in fibrosis or reflects the architectural changes associated with necro-inflammatory activity, such as cellular infiltration, tissue necrosis, or tissue oedema, is currently unknown. Furthermore, there remains the possibility that the higher liver stiffness measurements seen during an acute hepatitis flare may be artefactual, due to higher water content at the time of severe flares, rather than a true increase in liver stiffness. There is likely a complex interplay of MMPs and TIMPs during the phase of acute viral hepatitis which may influence liver stiffness at the time of inflammation (Koulentaki et al., 2002).

The true nature of the increased liver stiffness during a hepatitis flare can only be delineated by liver biopsy. We report 5 biopsies performed at the time of hepatitis B flare, showing a higher liver stiffness value than expected for the stage of fibrosis on histology. In addition, the fact that liver stiffness was significantly reduced by as early as 1 month in our subgroup analysis, and after 3-6 months from flares, suggest that the increase in liver stiffness is not reflective of underlying fibrosis, and more as a result of inflammation. This is supported by the lack of significant fibrosis on liver histology in the current study despite having an elevated liver stiffness value, and also in another study (Sagir et al., 2008).

Further studies are required to determine whether liver stiffness is affected by moderate inflammatory activity (e.g. ALT 2-5 times upper limit of normal). In a recently published study, increasing liver stiffness correlated well with even smaller gradients of ALT, including <0.5 x ULN, 0.5-1 x ULN, and 1-2 X ULN (Fung, Lai, But et al., 2008). However, because of the high AUROC already achieved with transient elastography, such small increments in ALT are unlikely to affect the accuracy of the test, and smaller increments in liver enzymes may reflect underlying fibrosis rather than inflammation.

There were several limitations of the current study. Firstly, the sample size were small, however, this is because the overall number of admissions for severe flares of CHB is relatively small. Despite the small sample size, the study was able to show significant liver stiffness changes over the different time points. Secondly, there were only a limited number of liver biopsies performed, as this is seldomly carried out in patients with well-documented evidence of hepatitis

B flares. In addition, over a third of the patients admitted for severe flares had elevated prothrombin time, precluding percutaneous biopsies.

In conclusion, liver stiffness is increased in CHB patients with severe flares, with return to near normal levels by six months. We recommend measuring liver stiffness to document liver fibrosis or cirrhosis in patients having severe hepatitis B flares should be postponed to at least 6 months after flare.

Chapter VII

EFFECT OF MILD ALT ELEVATION ON LIVER STIFFNESS MEASUREMENT IN CHRONIC HEPATITIS B

Introduction

In the previous chapter we have shown that in acute severe flares of hepatitis B, as defined by ALT level of greater than 10x ULN, liver stiffness values can be increased significantly, and subsequently decrease to normal levels once the ALT returns to normal ranges after resolution of the flare. Histological specimens obtained during the time of flare also showed that the liver stiffness values were spuriously higher than expected for the corresponding stage of fibrosis in our previous study and also in other recent studies (Arena et al., 2008; Coco et al., 2007). The mechanism in which liver stiffness is increased at the time of severe flare of hepatitis remains to be determined.

Whether lesser degree of hepatitis or inflammatory activity can also increase liver stiffness values is unknown. Our large population study of CHB subjects in chapter IV showed a positive correlation with liver stiffness with even small increments of ALT levels, suggesting that even mild inflammation can increase the level of liver stiffness (Fung, Lai, Fong et al., 2008). This is important as the ALT at the time of liver stiffness measurement may determine its accuracy in estimating the level of underlying fibrosis.

In the present study we aimed to investigate the effect of mild-to-moderate elevations of ALT on liver stiffness in patients with CHB.

Patients and Methods

Patients with CHB with ALT levels between 1-10x ULN who opted for antiviral treatment, seen at the Hepatitis Clinic, Queen Mary Hospital, Hong Kong, between the period of February 2008 and March 2009, were recruited. The ULN for ALT levels in males and females were defined as 53 and 31 U/L respectively (the ULN for the hospital laboratory at the time of the study).

Patients co-infected with HCV, HIV, or with other co-existing liver diseases such as Wilson's disease, autoimmune hepatitis, PBC, and alcoholic liver disease, were excluded. All patients underwent liver biopsy and liver stiffness measurement for assessment of liver fibrosis on the same day prior to starting antiviral therapy. Patient demographics and laboratory parameters were recorded at the time of liver stiffness measurement, including age, sex, liver biochemistry, HBsAg, HBeAg, anti-HBe, and serum HBV DNA levels. Oral antiviral therapy was commenced after liver biopsy and liver stiffness measurement were performed. The patients were followed up monthly until normalization of ALT. Once ALT normalization was achieved, a repeat liver stiffness measurement was performed. Written and verbal informed consent was obtained for liver biopsies and liver stiffness measurements respectively. This study has been approved by the Institutional Review Board of the University of Hong Kong.

Liver stiffness measurement

As described above, liver stiffness measurement was performed using transient elastography (Fibroscan) at 2 time-points. The first liver stiffness measurement was taken on the day of liver

biopsy. The second liver stiffness measurement was performed once normalization of ALT during the follow-up was achieved after commencement of antiviral therapy. This procedure has been well described in the preceding chapter on transient elastography. Patients with success rate of less than 50%, or an inter-quartile range-to-liver stiffness ratio of over 0.30 were excluded. Liver stiffness scores were expressed in units of kilopascals (kPa). A cut-off level of 11.0 kPa was used for liver cirrhosis, as derived from a previous study on CHB patients (Marcellin et al., 2009).

Liver biopsy

All patients in the current study underwent percutaneous liver biopsy immediately after the first liver stiffness measurement. Liver biopsy was performed using 16G Menghini needle. The necro-inflammatory scores and degree of fibrosis were assessed according to the modified HAI grading and staging system (Ishak et al., 1995; Knodell et al., 1981).

Statistical analysis

All statistical analyses were performed using the SPSS version 14.0 (SPSS Inc, Chicago, IL). Chi-squared test was used for categorical variables, and Fisher's exact test when appropriate. Paired related data were analyzed using the Wilcoxon paired test. Continuous variables with skewed distribution were analyzed using Mann-Whitney test. Continuous variables in three or more groups were analyzed using Kruskal-Wallis test. The diagnostic accuracy of liver stiffness

measurement was assessed using the AUROC curve. A p-value of <0.05 was considered statistically significant.

Results

A total of 52 patients were recruited into the study. Fourteen patients were excluded because the liver stiffness measurements were suboptimal according to the study criteria either at the time of liver biopsy (first time-point), or at the time of ALT normalization (second time-point). The remaining 38 patients were included in the final analysis, of which 21 were male, with a median age of 39 years (range, 18-63). The demographics and laboratory data are summarized in table 7.1. All were treatment-naïve at the time of study enrollment. Fourteen (37%) patients were treated with adefovir 10 mg daily, and 24 (63%) were treated with clevudine 30 mg daily.

All patients achieved normalization of ALT after commencement of antiviral therapy, and the median time between the first and second liver stiffness measurement was 3 months (range, 1-7). The median liver stiffness measurement values before antiviral therapy with elevated ALT levels and after antiviral therapy with normalization of ALT are shown in table 7.2.

Table 7.1 Basic demographic and laboratory data of patients

Parameters	Value
Total (n)	52
Suboptimal fibroscan (n)	14
Number included in study (n)	38
Male sex	21 (55%)
Age (years)	39 (18-63)
Baseline parameters	
Bilirubin (umol/L)	14 (6-27)
ALT (U/L)	89 (46-501)
AST (U/L)	57 (27-255)
Albumin (g/L)	47 (42-53)
Platelets (x 10^9)	207 (102-310)
INR	1.0 (0.9-1.3)
HBeAg-positive	15 (39%)
Histology	
HAI Activity	5 (1-12)

Fibrosis	1 (0-3)
F0	5 (13%)
F1	22 (58%)
F2	7 (18%)
F3	4 (11%)

* Continuous variables expressed as median values (range). HAI=modified hepatic activity index.

Table 7.2 Liver stiffness parameters at the 2 different time-points

Parameters	Value
Elevated ALT before antiviral therapy	
Liver stiffness (kPa)	9.2 (4.5-34.3)
Interquartile range (IQR)	1.2 (0.1-5.8)
IQR to kPa ratio	0.14 (0-0.3)
Success rate	100% (50-100)
Normal ALT after antiviral therapy	
Liver stiffness (kPa)	7.0 (3.5-14.6)
Interquartile range (IQR)	1.1 (0-3.2)
IQR to kPa ratio	0.16 (0-0.3)
Success rate	100% (63-100)
Median time between measurements (months)	3 (1-7)

* Continuous variables expressed as median values (range)

Liver stiffness before and after ALT normalization

There was a significantly lower median liver stiffness measurements after the commencement of antiviral therapy with the normalization of ALT levels, compared to pre-treatment levels in the 38 patients (7.0 vs 9.2 kPa respectively, $p<0.001$). Further analysis was performed by stratifying the patients into 3 different pre-treatment ALT groups: 1-2x ULN, 2-5x ULN, and 5-10x ULN. There was a significant decline in median liver stiffness after the normalization of ALT levels, compared to patients with pre-treatment ALT 2-5x ULN (7.5 vs 9.2 kPa respectively, $p=0.028$) and 5-10x ULN (6.6 vs 10.9 kPa respectively, $p=0.008$). In patients with pre-treatment ALT of 1-2x ULN, there was a trend for lower liver stiffness measurements after ALT normalization compared to pre-treatment measurements (6.6 vs 7.3 kPa respectively, $p=0.055$). The overall results are summarized in figure 7.1.

The overall median difference in liver stiffness between the two different time-points, and also stratified by their pre-treatment ALT of 1-2x ULN, 2-5x ULN and 5-10 x ULN are shown in figure 7.2. The median liver stiffness reduction observed in patients with pre-treatment ALT of 1-2x ULN, 2-5X ULN, and 5-10x ULN were 0.9, 1.4, and 3.9 kPa respectively, with an overall reduction of 2.0 kPa. The higher the pre-treatment ALT, the higher the liver stiffness reduction observed ($p=0.027$). The results suggest that the reduction of liver stiffness after ALT normalization is greater for those with higher pre-treatment ALT levels.

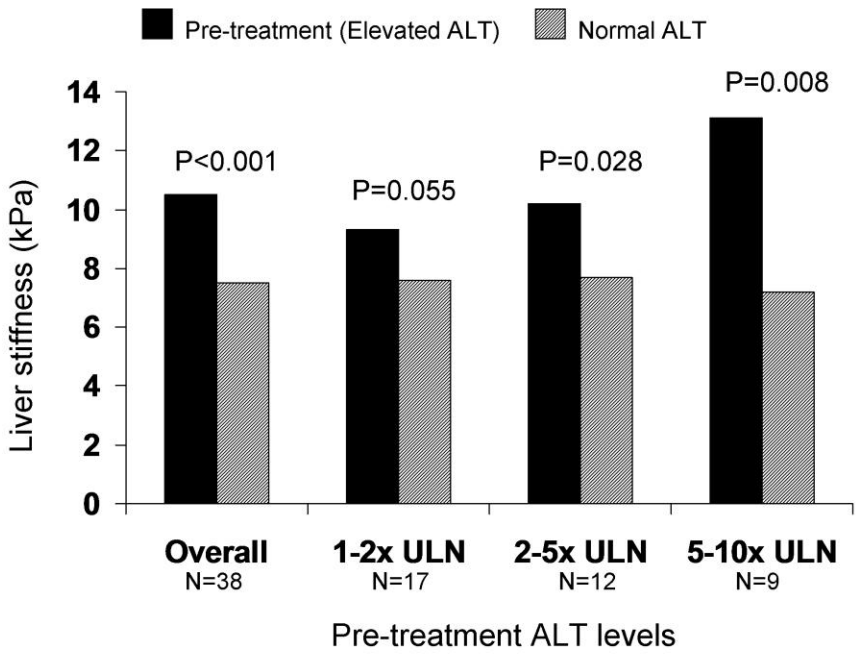

Figure 7.1 Difference in liver stiffness measurements at pre-treatment and after normalization of ALT, stratified by pre-treatment ALT levels

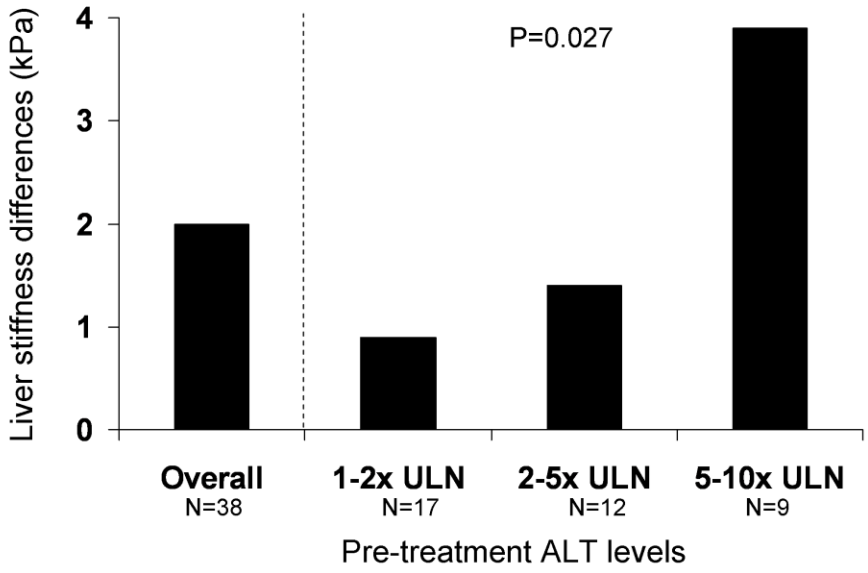

Figure 7.2 Median liver stiffness differences between pretreatment liver stiffness measurements and after ALT normalization, stratified by pre-treatment ALT levels

Accuracy of liver stiffness measurement: Elevated versus normal ALT

The liver stiffness measurements obtained at the 2 different time-points were compared for accuracy. The AUROC curve for diagnosing F2 fibrosis in patients with elevated ALT was 0.68, compared with 0.73 for the same patients after ALT normalization (Figure 7.3).

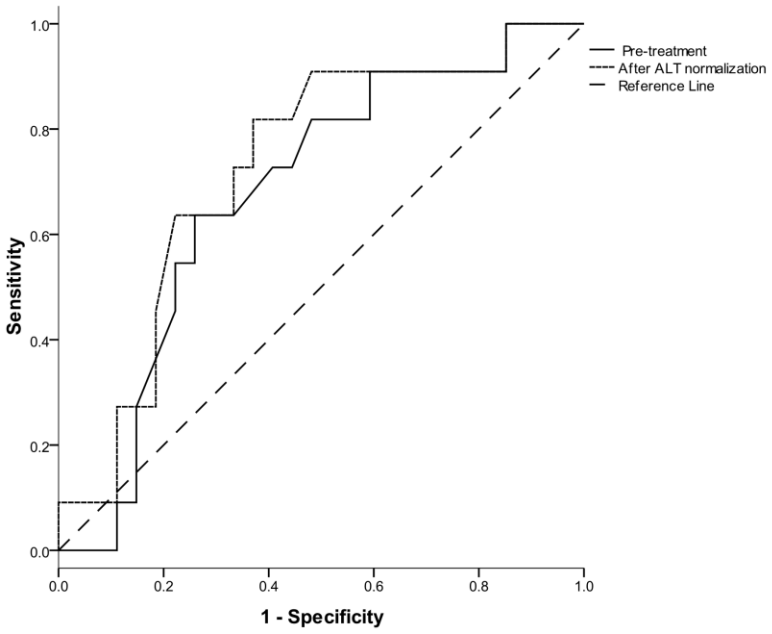

Figure 7.3 Receiver operator characteristic curve for diagnosis of F2 fibrosis using liver stiffness measurement with ALT elevation (pre-treatment) and after ALT normalization (post-treatment)

None of the 38 patients had evidence of cirrhosis on liver histology. Using the cutoff value of 11.0 kPa for cirrhosis in CHB patients, 12 (32%) patients would have been misclassified as having cirrhosis using liver stiffness measurements taken at the time of ALT elevation. In contrast, 6 (16%) patients would have been misclassified as having cirrhosis using liver stiffness measurements at the time where ALT had normalized.

Discussion

Transient elastography has been shown to be an excellent non-invasive test for assessment of liver fibrosis in numerous studies (Castera et al., 2005; Foucher, Chanteloup et al., 2006; Friedrich-Rust et al., 2008; Ziol et al., 2005). However, the diagnostic accuracy of this test needs to be further refined by identifying factors which may affect liver stiffness results other than the presence of fibrotic tissue. Our previous study in the preceding chapter have shown that large fluctuations in ALT, such as those seen with severe flares of hepatitis B (as defined by ALT >10x ULN), can increase the liver stiffness measurements determined by transient elastography. This has also been observed in other studies of acute severe hepatitis (Arena et al., 2008; Coco et al., 2007). The exact mechanism for the increased liver stiffness is not known. Whether the artefactual increase is due to increase oedema, cellular infiltration, or other changes incited by liver inflammation requires further studies

A previous study of CHB patients without histological data showed significantly higher liver stiffness values even with smaller incremental ALT gradients of <0.5x ULN, 0.5-1x ULN, 1-2x ULN, and 2-5x ULN (Fung, Lai, Fong et al., 2008). There is no study which examines and compares the liver stiffness measurement and liver histology in patients with mild-to-moderate elevation of ALT at the first time point, with follow-up of these patients at the second time point when ALT levels are normalized. It is an important issue because mild-to-moderate elevations of ALT constitute over 36% of our CHB population (Yuen et al., 2005). In the present study, we described the effect of mild-to-moderate elevation of ALT (<10x ULN) on liver stiffness measurement. Paired liver stiffness measurements were obtained, at the time of ALT elevation

(pre-treatment), and after ALT normalization (treatment). There was a significant decline in liver stiffness measurements once normalization of ALT occurred. The median time between the 2 liver stiffness measurements was short at 3 months. This would suggest that the decline in liver stiffness was due to a decline in inflammatory activity rather than to regression of fibrosis stage.

The current study also raises the question of the optimal timing to perform transient elastography. For patients with severe flares, the advice would be to wait until ALT is nearly normal for a period of time before transient elastography is performed. For mild-to-moderate elevation of ALT, the question arises whether transient elastography should be performed at a time where ALT is normal or near-normal, or to adopt an algorithm which will stratify the different ALT levels with different liver stiffness cut-off values corresponding to the different fibrosis stages. A recent published ALT-based algorithm has been devised for CHB patients, using a higher liver stiffness values for optimal cut-offs in patients with elevated ALT (Chan, Wong, Choi et al., 2009). However, given the non-invasive nature and the ease of performing transient elastography, one could recommend patients to have a repeat liver stiffness measurement performed if ALT normalization is achieved.

Furthermore, the diagnostic accuracy of transient elastography in the current study appears to be improved with normal ALT levels. The AUROC analysis was performed using F2 rather than a higher fibrosis stage as all patients in the study had F3 or less, and the number of patients with F3 fibrosis would not have been sufficient for an adequate analysis. Using F2 or greater fibrosis stage to assess the diagnostic accuracy of transient elastography, the AUROC was able to be

improved from 0.68 to 0.73 after ALT normalization. Transient elastography has been shown to be an excellent test in excluding severe fibrosis and cirrhosis (personal experience), but performs less well in predicting severe fibrosis and cirrhosis. By delaying liver stiffness measurements until the time of ALT normalization, the false-positive results for diagnosing liver cirrhosis was halved from 32% to 16% in the present study, and may possibly even decline further with longer period of ALT normalization.

There were several limitations of the current study. The total number of patients was small, but despite this, the study was able to show a significant decline in liver stiffness even when stratified by different pre-treatment ALT levels to smaller groups. Ideally, a second biopsy at the time of ALT normalization with repeat transient elastography would provide definitive evidence of the role of mild-to-moderate inflammation on liver stiffness measurements. However, the decrease in liver stiffness after a median of 3 months suggested that it is likely due to non-structural and easily reversible factors such as inflammation with oedema. Repeating a second liver biopsy at such close intervals was not possible for the current study.

In conclusion, even mild to moderate elevation in ALT levels may increase liver stiffness measurements independent of underlying liver fibrosis. Higher ALT levels were associated with higher discrepancies in liver stiffness. Therefore, the timing of liver stiffness measurement is important, and in cases where ALT is persistently elevated, use of algorithms correcting for the ALT levels is recommended.

Chapter VIII

CORRELATION OF LIVER HISTOLOGY IN PATIENTS WITH A SPECTRUM OF HEPATITIS B-RELATED LIVER DISEASE

Introduction

The rationale for transient elastography is based on the theory that liver stiffness is positively correlated with the amount of fibrotic tissue within the liver. It is well established that the cut-off liver stiffness values used for different fibrosis stages are dependent on the underlying disease. However, there is increasing evidence to suggest that even within the same disease, the optimal cut-off values for different degrees of fibrosis may vary with the underlying inflammatory activity, as reflected by higher levels of serum ALT (Chan, Wong, Choi et al., 2009). In patients with CHB, the inflammatory activity and its surrogate marker, ALT level, can fluctuate during the course of the disease. Our previous studies in the preceding chapters have shown that underlying inflammatory activity can increase liver stiffness values, causing an overestimation of the degree of fibrosis in those with mild-to-moderate and severe degrees of hepatitis.

In the current study, we determined liver stiffness values and histological features in subjects with a wide spectrum of HBV-related liver disease and in healthy individuals. We compared the liver stiffness in subjects with no known liver disease, occult hepatitis B infection, active CHB, and hepatitis B-related cirrhosis.

Patients and Methods

The present study recruited a total of 187 subjects from Queen Mary Hospital, University of Hong Kong, Hong Kong, between the periods of November 2005 to November 2008. There were 4 groups of subjects (28 healthy subjects, 18 occult hepatitis B patients, 121 active CHB patients, and 20 end-stage cirrhosis patients). The group without known underlying liver disease (healthy subjects) was living-related donors recruited from the liver transplant program at Queen Mary Hospital. Subjects in this group were negative for HBsAg, antibody to HCV, significant alcohol intake history (as defined by an intake of >20g/day), or any other known liver diseases. Patients with occult hepatitis B infection were recruited from healthy blood donors who were negative for HBsAg, but positive for anti-HBc with detectable HBV DNA (method described below). Patients with active CHB were recruited from the hepatitis clinic at Queen Mary Hospital. These patients were positive for HBsAg for over 6 months, with elevated ALT and HBV DNA levels >20,000 IU/mL. The hepatitis B-related cirrhosis group was liver transplant recipients recruited from the liver transplant program. Written and verbal consent was obtained for liver biopsy and liver stiffness measurements respectively. This study has been approved by the Institutional Review Board of the University of Hong Kong.

Liver histology

All patients included in the current study had liver histology available. Liver biopsy was performed on the patient groups with occult hepatitis B and active CHB using a 16G Menghini needle after written informed consent. The liver histology was graded using the modified HAI score.(Ishak et al., 1995; Knodell et al., 1981) In the groups with normal subjects and HBV-

related cirrhosis, histology was obtained at the time of liver transplantation from the donor (intra-operative biopsy specimen) and from the recipient (explant specimen).

Liver stiffness measurements

Liver stiffness was measured using transient elastography (Fibroscan, Echosens, Paris, France). All patients had transient elastography performed either on the same day or within one week of obtaining the liver histology. Patients who had a success rate of <50%, IQR-to-liver stiffness ratio of >30%, or less than 10 validated measurements, were excluded.

Liver biochemistry and viral load

Complete blood count, coagulation profile, and routine liver biochemistry were determined at the time of liver biopsy or surgery. In patients with active CHB, the HBV DNA levels were determined using the Cobas Taqman assay, with a lower limit detection of 12 IU/mL (Roche Diagnostics, Branchburg, NJ). For detection of occult hepatitis B in blood donors, HBV DNA was extracted from 500 μL of serum using the QIAamp DSP Virus Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, with a final elution of 26 μL. HBV DNA in the extracts was then quantitatively measured by the Artus HBV RG PCR Kit (Qiagen Hamburg GmbH, Hamburg, Germany), using the Rotor-Gene 3000 Real-time Multiplexing System (Corbett Research, Mortlake, Australia), according to the manufacturer's instructions. This test has a 95% lower limit of quantitation of 3.8 IU/mL of serum and a linear range of detection from 1.1 IU/mL to 4×10^9 IU/mL of serum. Since the serum HBV DNA levels of these

occult HBV subjects were very low, in order to eliminate the chance of false positive or negative results, Artus HBV test was performed 3 times. Genuine HBV DNA-positive result was defined as having ≥2 out of 3 positive HBV DNA signals by 3 independent Artus test runs.

Statistical analysis

All statistical analyses were performed using the SPSS version 14.0 (SPSS Inc, Chicago, IL). Continuous variables with skewed distribution were analyzed using Mann Whitney test. Related data were analyzed using the Wilcoxon paired test. Continuous variables with more than 2 independent samples were analyzed using the Kruskal-Wallis test. Correlation between liver stiffness and liver biochemistry and histology score was performed using Spearman's bivariate correlation. Multivariate analysis was performed using multiple regressions on variables significant on univariate analysis. The receiver-operating characteristic (ROC) curves and AUROC were calculated. The optimal cut-off values were defined as the value giving the highest sensitivity and specificity. A p-value of <0.05 was considered statistically significant.

Results

A total of 187 patients had both liver biopsy and transient elastography performed during the study period. Of these, thirty (16%) patients were excluded because of suboptimal liver stiffness measurements. Of these suboptimal measurements, eleven patients were liver recipients with very small livers and ascites, accounting for the overall higher-than-expected failure rate of liver stiffness measurements. The remaining 19 patients were excluded due to suboptimal measurements secondary to overlying adipose tissue. One hundred and fifty-seven patients were included in the final analysis. The demographics and laboratory data are summarized in table 8.1.

Healthy subjects

Twenty-eight liver donors were recruited as healthy subjects, with a median age of 32 years (range, 18-56) and ALT of 16 U/L (range, 4-51). None of the healthy subjects had fibrosis on liver histology. The median liver stiffness was 4.6 kPa (range, 2.0-7.1). Twenty-six (93%) subjects had liver stiffness of less than 6.0 kPa. All healthy subjects had liver stiffness values of less than 7.2 kPa, the optimal cut-off for F2 as defined in a previous study of CHB patients (Marcellin et al., 2009).

Table 8.1 Baseline demographics of study population

Parameter	Value
Total patients	187
Invalid liver stiffness measurements	30 (16%)
Number in final analysis	157
Male gender	101 (64%)
Age (years)	41 (18-63)
Groups	
Healthy subjects	28(18%)
Occult hepatitis B	18 (11%)
Active chronic hepatitis B	102 (65%)
End-stage hepatitis B cirrhosis	9 (6%)

Continuous variables are shown as median values, with range in brackets.

Occult hepatitis B

Eighteen patients with evidence of occult hepatitis B infection were included, with a median age of 47 years (range, 20-59) and ALT of 24 U/L (range, 8-48). Fourteen (78%) patients had no fibrosis on liver biopsy. The remaining 4 (22%) patients had minimal stage 1 fibrosis. The median liver stiffness in patients with occult hepatitis B was 4.2 kPa (range, 3.4-6.9), with all patients having liver stiffness values of less than 7.2 kPa.

Active chronic hepatitis B

One hundred and two patients had active CHB, with a median age of 41 years (range, 18-63). The median ALT was 89 U/L (range, 46-501). The median liver stiffness was 8.7 kPa (range, 3.6-44.3). Thirty-two (31%) patients had liver stiffness value >11.0 kPa, the optimal cut-off value for cirrhosis defined in a previous study of CHB patients (Marcellin et al., 2009) Of these 32 patients, 12 (38%) had minimal fibrosis (stage 0-1), and a further 16 (50%) patients had moderate fibrosis (stage 2-3). The remaining 4 (12%) patients had histological cirrhosis with HAI fibrosis stage 5-6. In active CHB, using liver stiffness measurements to predict cirrhosis had a sensitivity of 100%, specificity of 69%, a positive predictive value of 10%, and a negative predictive value of 100%.

Of these 102 patients with chronic active hepatitis B, 15 patients underwent repeat liver biopsies with valid liver stiffness measurements at 12 months after commencing oral antiviral therapy with subsequent normalization of ALT. None of these patients had cirrhosis at either the first or second biopsy. However, 3 patients had liver stiffness measurement of >11.0 kPa indicating cirrhosis before treatment. Of these 3 patients, 2 had liver stiffness of <11.0 kPa (8.5 and 6.8 kPa) after 1 year of antiviral therapy with normalization of ALT. There was a significant decline in liver stiffness after ALT normalization compared to the time of active hepatitis (8.6 vs 6.0 kPa, p=0.001) without associated significant decline in fibrosis stages.

End-stage CHB cirrhosis

Nine patients had end-stage liver cirrhosis secondary to CHB requiring liver transplantation. The median age was 55 years (range, 51-59), with a median liver stiffness of 33.8 kPa (range, 11.9-75.0) and ALT of 91 U/L (range, 16-324). All patients had liver stiffness value of >11.0 kPa, the cut-off for cirrhosis in CHB patients.

Comparison of groups

The demographic and laboratory data of the four different groups are shown in table 8.2. The liver stiffness value of the study population is summarized in figure 8.1 according to their groups. There was no significant difference in median liver stiffness between healthy subjects and occult hepatitis B (4.6 vs 4.2 kPa respectively, p=0.796). Patients with active CHB had a significantly higher median liver stiffness compared with occult hepatitis B patients (8.7 vs 4.2 kPa respectively, p<0.001) and healthy subjects (8.7 vs 4.6 kPa respectively, p<0.001). In patients with minimal (F1) fibrosis in the occult hepatitis B and active CHB groups, there was a trend for higher liver stiffness measurement in the active CHB group (5.2 vs 7.0 kPa respectively, p=0.066). Patients with end-stage CHB cirrhosis had a significantly higher median liver stiffness compared with active CHB patients (33.8 vs 8.7 kPa respectively, p<0.001), occult hepatitis B patients (33.8 vs 4.2 kPa respectively, p<0.001), and healthy subjects (33.8 vs 4.6 kPa respectively, p<0.001).

Table 8.2 Demographics and laboratory values of normal subjects, occult hepatitis B, active chronic hepatitis B, and hepatitis B-related cirrhosis

Parameter	Healthy Subjects N=28	Occult Hepatitis B N=18	Active Chronic Hepatitis B N=102	Hepatitis B Cirrhosis N=9
Age	32 (18-56)	47 (20-59)	41 (18-63)	55 (51-59)
Male sex	17 (61%)	14 (78%)	63 (62%)	7 (78%)
Bilirubin	9 (4-17)	8 (4-13)	13 (6-30)	28 (18-565)
ALT	16 (4-51)	24 (8-48)	89 (46-501)	91 (16-324)
AST	18 (11-40)	26 (12-40)	55 (27-255)	110 (29-512)
Albumin	45 (38-50)	45 (41-49)	47 (41-54)	33 (22-45)
Platelets	246 (173-355)	236 (155-365)	210 (102-334)	64 (33-189)
INR	0.9 (0.8-1.1)	0.9 (0.7-1.0)	1.0 (0.9-1.4)	1.4 (1.0-1.9)
HAI activity	0 (0-0)	1 (0-4)	5 (2-12)	4 (0-12)
Fibrosis stage	0 (0-0)	0 (0-1)	1 (0-6)	6 (6-6)
F≤2	28 (100%)	18 (100%)	88 ((86%)	0 (0%)
F3-4	0 (0%)	0 (0%)	10 (10%)	0 (0%)
F5-6	0 (0%)	0(0%)	4 (4%)	9 (100%)
Liver stiffness	4.6 (2.0-7.1)	4.2 (3.4-6.9)	8.7 (3.6-44.3)	33.8 (11.9-75)

Units: age in years, bilirubin in umol/L, ALT in U/L, AST in U/L, albumin in g/L, platelets in 10^9/L, fibrosis stage in HAI, liver stiffness in kPa. Continuous variables are displayed as median values. HAI=histology activity index, ALT=alanine aminotransferase, AST=aspartate aminotransferase, INR=international normalized ratio

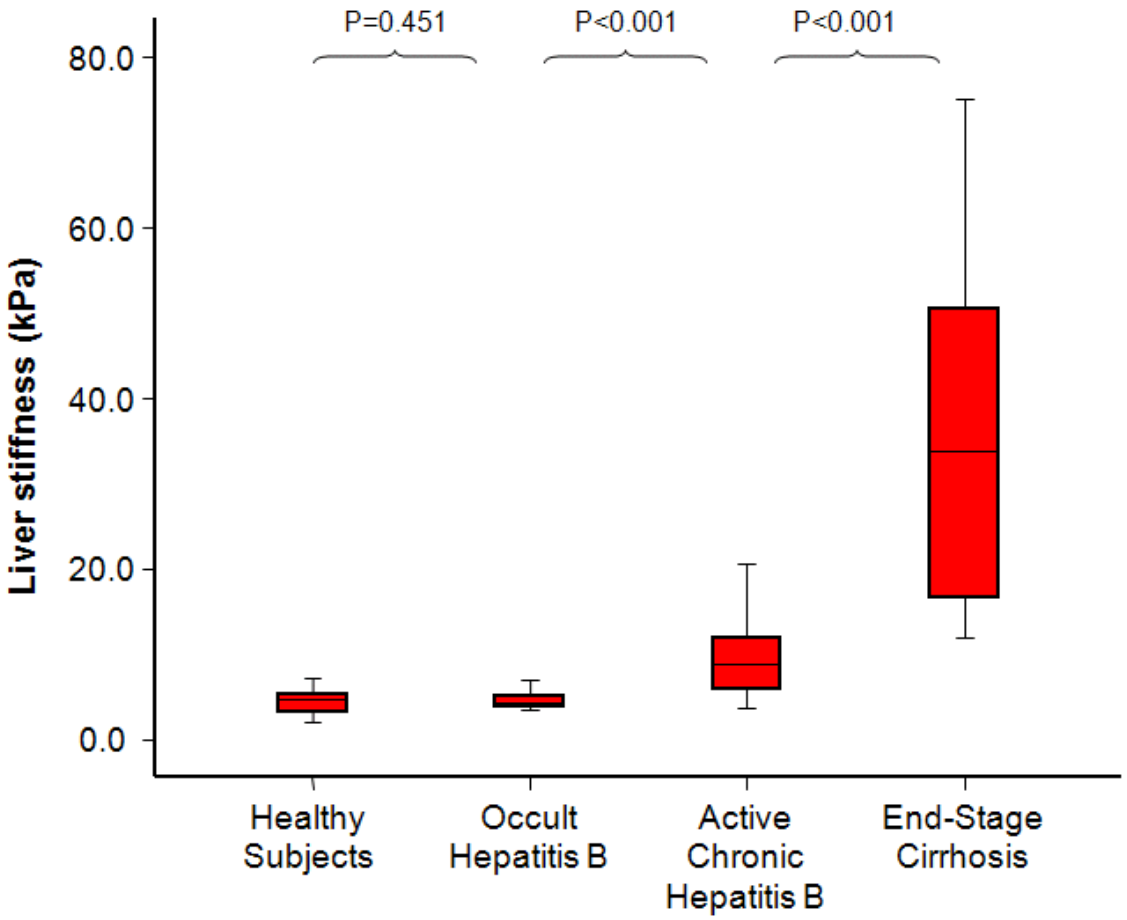

Figure 8.1 Liver stiffness measurements in healthy subjects, occult hepatitis B, active chronic hepatitis B, and end-stage liver hepatitis B cirrhosis

Relationship between liver stiffness and biological parameters

The correlation between liver stiffness and biological parameters is shown in table 8.3. There were significant correlations with age, necro-inflammatory activity, histological fibrosis, bilirubin, ALP, ALT, AST, GGT, platelets, and INR. After stratification into different age groups and levels of ALT and platelets, there were higher liver stiffness measurements in patients with higher age groups, higher ALT levels, and lower platelet counts (Table 8.4). After multivariate analysis, histological fibrosis, ALT, AST, GGT, and platelets remains significantly correlated with liver stiffness.

Diagnostic performance

The AUROC, optimal cut-offs, sensitivity and specificity for diagnosing stage 2, 3, and ≥4 fibrosis for our study population is shown in table 8.5. The ROC curves for stage 2, 3, and ≥4 fibrosis are shown in figure 8.2.

Table 8.3 Correlation between liver stiffness and demographic and laboratory data

Parameter	Correlation co-efficient	P value
Age	0.204	0.010
HAI activity	0.626	<0.001
HAI fibrosis	0.636	<0.001
Bilirubin	0.408	<0.001
ALP	0.342	<0.001
ALT	0.550	<0.001
AST	0.624	<0.001
GGT	0.397	<0.001
Albumin	0.021	0.794
Platelets	-0.513	<0.001
INR	0.531	<0.001

* HAI=histology activity index, ALP=alkaline phosphatase, ALT=alanine aminotransferase, AST=aspartate aminotransferase, GGT=gamma-glutamyl transferase, INR=international normalized ratio

Table 8.4 Liver stiffness stratified by age groups, and levels of ALT and platelets

Parameter	Numbers	Liver stiffness (kPa)	P value
Age (years)			0.027
<35	61	5.6 (2.4-34.3)	
36-55	74	7.3 (2.0-75.0)	
>55	22	8.7 (3.3-49.6)	
ALT			<0.001
<1x ULN	50	4.7 (2.0-75.0)	
1-2x ULN	52	8.6 (3.4-44.3)	
>2x ULN	55	9.5 (4.5-75.0)	
Platelets ($x10^9$/L)			<0.001
<150	23	16.0 (5.3-75.0)	
150-250	94	6.8 (2.0-34.3)	
>250	40	5.3 (2.7-20.5)	

* ALT=alanine aminotransferase, ULN=upper limit of normal.

Table 8.5 The AUROC and optimal cut-off values for liver stiffness measurement in diagnosing stage 2, 3, and 4 or more fibrosis.

	Fibrosis Stage ≥2	Fibrosis Stage ≥3	Fibrosis Stage ≥4
AUROC	0.87	0.89	0.89
Cut-off (kPa)	9.4	9.9	11.3
Sensitivity	81%	91%	93%
Specificity	82%	80%	82%

* AUROC=area under the receiver operating characteristic curve, kPa=kilopascals

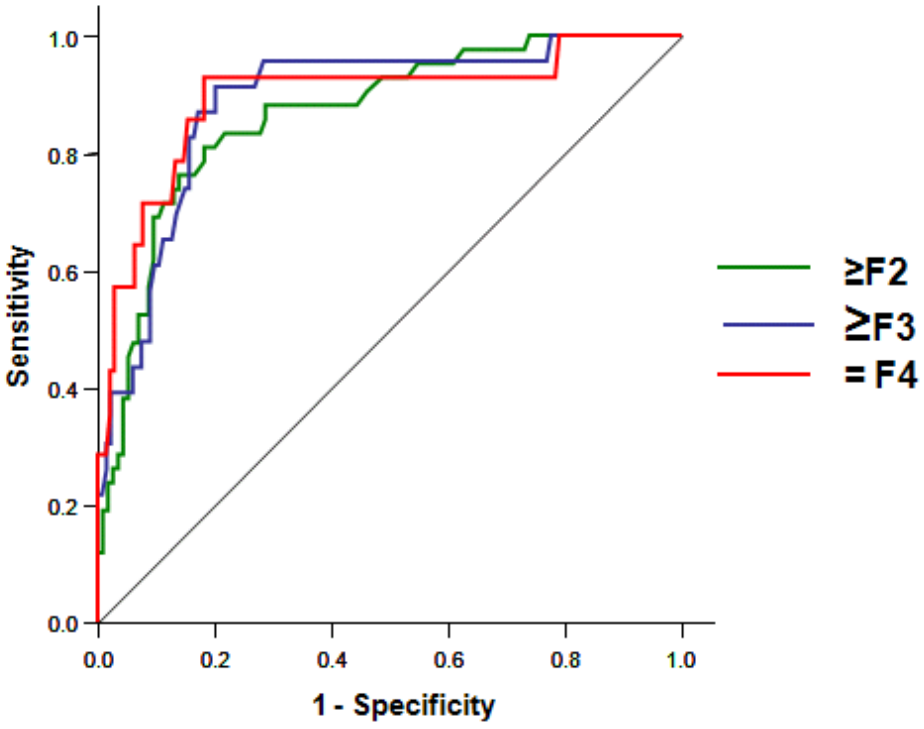

Figure 8.2 Receiver operator characteristic curve for liver stiffness and greater than stage 2 fibrosis (F2), stage 3 fibrosis (F3), and cirrhosis (F4)

Discussion

In the current study, we determined the liver stiffness of healthy subjects and patients at different ends of the spectrum of hepatitis B-related diseases ranging from occult infection to end-stage cirrhosis. Although the diagnostic accuracy of transient elastography appeared excellent with an AUROC of 0.89 for stage 3 and 4 fibrosis, the performance was dependent on the underlying disease activity. In both healthy subjects and occult hepatitis B patients (none had histological grade of F2, F3 or F4 fibrosis), all subjects had liver stiffness values of <7.1 kPa. There were no false positive liver stiffness results indicating advance fibrosis or cirrhosis. This suggests that liver stiffness of <7.1 kPa can be safely adopted as defining insignificant fibrosis. In patients with end-stage hepatitis B cirrhosis, there were no false negative results with transient elastography, with all subjects having liver stiffness values of >11.0 kPa.

In patients with active CHB, the diagnostic accuracy of transient elastography appeared to be dichotomous. It had an excellent negative predictive value of 100% in excluding cirrhosis. However, transient elastography had a poor positive predictive value of 10% in active CHB; 28 out of 32 patients with liver stiffness >11.0 kPa had no evidence of cirrhosis on histology. Therefore, in active CHB, additional test such as liver biopsy is required to confirm the presence of severe fibrosis or cirrhosis in patients with liver stiffness >11.0 kPa. Alternatively, repeating transient elastography after normalization of ALT (eg: after 6 months) may provide a more accurate liver stiffness measurement.

Although our previous study have shown that severe necro-inflammatory activity (as defined by ALT>10x ULN) can affect liver stiffness, the current study suggests that a much lesser degree of inflammation may also increase liver stiffness, and therefore reduce the accuracy of transient elastography. These results are also in accordance with our previous large population study which have shown that even milder degrees of ALT elevation are associated with significantly higher median values of liver stiffness (Fung, Lai, Fong et al., 2008), and in the preceding study showing the effect of mild-to-moderate ALT elevations on liver stiffness.

In the present study, a cut-off level of liver stiffness of 11.3 kPa for cirrhosis is associated with high sensitivity and specificity (93% and 82% respectively) for the overall group. This is in accordance with another validating study of CHB patients in which the sensitivity and specificity for diagnosing cirrhosis using a cut-off of 11.0 kPa were 93% and 87% respectively.(Marcellin et al., 2009) The low positive predictive value of 11.0 kPa in the present study for the group with active hepatitis can be explained by the different population studied. The median ALT of the current study was higher than that of the previous study (89 vs 54 U/L respectively). In addition, after multivariate analysis, both ALT and AST were significantly associated with liver stiffness in addition to histological fibrosis and platelet levels, whereas only histological fibrosis and platelet levels were significantly associated with liver stiffness in the previous validation study. A recent study on CHB patients has also shown that patients with the same fibrosis stage albeit higher ALT levels tend to have higher liver stiffness values, and the diagnostic performance of transient elastography is reduced (Chan, Wong, Choi et al., 2009). It emphasizes the role of abnormal ALT on liver stiffness measurement.

Transient elastography remains one of the most promising non-invasive techniques in assessing liver fibrosis. As this technology matures with increasing experience, we are now beginning to understand the finer intricacies of measuring liver stiffness. The current study shows that liver stiffness measurement is not accurate in diagnosing cirrhosis in patients with active hepatitis and elevated ALT using the current cut-off values. Unfortunately, these patients are also a group in which clinicians are very interested to know whether underlying advance fibrosis or cirrhosis is present, so that the decision to start antiviral therapy can be made. To increase the diagnostic accuracy of transient elastography, the optimal timing of performing a scan needs to be established, that is, whether it should be performed (or repeated) after ALT is normalized. An alternative approach would be to adopt different cut-off values depending on the ALT, or to combine liver stiffness values into a more complex model, including markers of inflammation, to improve the diagnostic accuracy.

One limitation of the current study was that even though the study had a wide spectrum of disease severity, further useful information can be gained with the availability of patients with inactive CHB, and with the inclusion of more patients with well-compensated cirrhosis. Nevertheless, the current study was able to demonstrate the performance of transient elastography in a wide range of disease severity. Furthermore, the current study provides histological correlation with liver stiffness for the first time in healthy patients and in patients with occult hepatitis B. The median liver stiffness in healthy subjects in the current study was similar to the mean level of liver stiffness in an earlier study of healthy subjects without

histological correlation (4.6 and 5.5 kPa respectively) (Roulot et al., 2008). Another limitation of the study is that the body weight and body mass index was not measured, which may affect the liver stiffness measurements.

In conclusion, liver stiffness measurement has an overall good diagnostic accuracy, particularly with an excellent negative predictive value in patients with CHB. However, in patients with active hepatitis and elevated ALT, measuring liver stiffness has a poor positive predictive value, and further studies are required to determine both the optimal timing for performing transient elastography, and the optimal cut-off values for different levels of inflammatory activity.

Chapter IX

LONG-TERM PROGNOSTIC SIGNIFICANCE OF LIVER STIFFNESS MEASUREMENT IN CHRONIC HEPATITIS B

Introduction

Several important risk factors have been associated with the development of HCC, including older age, male sex, delayed HBeAg seroconversion, and higher levels of HBV DNA (C. J. Chen et al., 2006; Chu, Hung, Lin, Tai, & Liaw, 2004; Chu & Liaw, 2007). One of the major risk factors for the development of HCC is the presence of advanced fibrosis or cirrhosis, with the latter being the most important factor for hepatocarcinogenesis in patients with chronic liver disease from a wide range of etiology (V. T. Nguyen, Law, & Dore, 2009; Yuen et al., 2009; Yuen, Tanaka et al., 2008; Yuen et al., 2005).

Currently, measuring liver stiffness is a relatively new technology, and there is a paucity of data regarding the long-term prognostic application of transient elastography in patients with chronic liver diseases. In the only large prospective study of long term prognosis with transient elastography in chronic hepatitis C patients to date, higher liver stiffness was shown to be associated with the development of HCC (Masuzaki et al., 2009). Chronic hepatitis C-related HCC usually occurs with underlying cirrhosis. However, a significant proportion of CHB patients (approximately 20-30%) may not have established cirrhosis because the HBV is an oncogenic virus (Fung, Lai, & Yuen, 2009). Treatments with antiviral agents have been shown to improve survival and decrease the chance of liver-related complications in cirrhotic and non-cirrhotic patients (Liaw et al., 2004a; Yuen et al., 2007). Identifying high-risk patients for treatment is therefore of paramount importance so that antiviral therapy may be commenced.

There is currently no large study investigating the prognostic value of liver stiffness measurement in patients with CHB. In the present study, we prospectively followed up a large cohort of HBeAg-negative CHB patients after an initial liver stiffness measurement to assess the use of transient elastography as a predictor of HCC development and mortality.

Patients and Methods

From January 2006 to December 2006, 651 patients with HBeAg-negative CHB underwent liver stiffness measurement using transient elastography. All patients were followed up at the Hepatitis and Liver Clinics in Queen Mary Hospital, Hong Kong. Patients co-infected with HCV, HIV, or with other co-existing liver diseases including Wilson's disease, autoimmune hepatitis, PBC, and alcoholic liver disease were excluded. All patients were positive for HBsAg for at least 6 months. This study was approved by the Institutional Review Board of the University of Hong Kong.

Patient follow-up

Patients were followed-up 3-6 monthly with regular liver biochemistry and AFP measurements. In patients with elevated AFP (>20ng/mL), an ultrasound, triphasic CT, or MRI scan was performed. HBV DNA was determined at the time of liver stiffness measurement. This was performed using the Cobas Taqman assay (Roche, Branchburg, NJ), with a lower limit of detection of 60 copies/mL.

Liver stiffness measurement

At least 10 valid measurements were obtained for each patient. Results were included in the final analysis only if the success rate was over 50%, and the IQR-to-liver stiffness ratio was less than 0.30. Those patients with liver stiffness measurements outside these criteria were excluded. The

median values of the validated measurements were representative of the liver stiffness and expressed in units of kilopascals (kPa). Informed consent was obtained from each patient.

Diagnosis of HCC

The diagnosis of HCC was established using a combination of elevated AFP, and typical findings on tri-phasic CT or MRI. The diagnosis was confirmed with histological specimens in patients who underwent surgical resection or liver transplantation, or with subsequent imaging showing tumour progression in non-resected patients.

Statistical analysis

All statistical analyses were performed using the SPSS version 17.0 (SPSS Inc, Chicago, IL). Chi-squared test was used for categorical variables, and Fisher's exact test when appropriate. Continuous variables with skewed distribution were analyzed using Mann-Whitney test. The cumulative incidences of HCC and survival analysis were performed using the Kaplan-Meier method, with log-rank testing for comparison. Multivariate analysis using Cox regression was performed to identify independent factors associated with HCC, mortality, and biochemical flares. A p-value of <0.05 was considered statistically significant.

Results

A total of 651 HBeAg-negative CHB patients underwent liver stiffness measurements in 2006. Of these, 116 (18%) were excluded due to invalid liver stiffness measurement as set out in the exclusion criteria. Of the 535 remaining patients, 1 patient was excluded since the patient already had HCC at the time when transient elastography was performed. Another 6 patients were excluded as they had no further follow-up at the clinic after the initial liver stiffness measurements. The patient recruitment algorithm is shown in figure 9.1. Five hundred and twenty-eight patients were included in the final analysis with a median age of 42 years (range, 18-86), of which 324 (61%) were male. The median follow-up time was 35 months (range, 3-42). One hundred and six patients received antiviral therapy during the follow-up period with a median treatment length of 36 months (range, 5-41). Of these, 72, 8, and 6 patients were treated with lamivudine, adefovir, and entecavir respectively. The remaining 20 patients were treated with a combination of these oral agents. The demographics and baseline laboratory results of the study population are summarized in table 9.1.

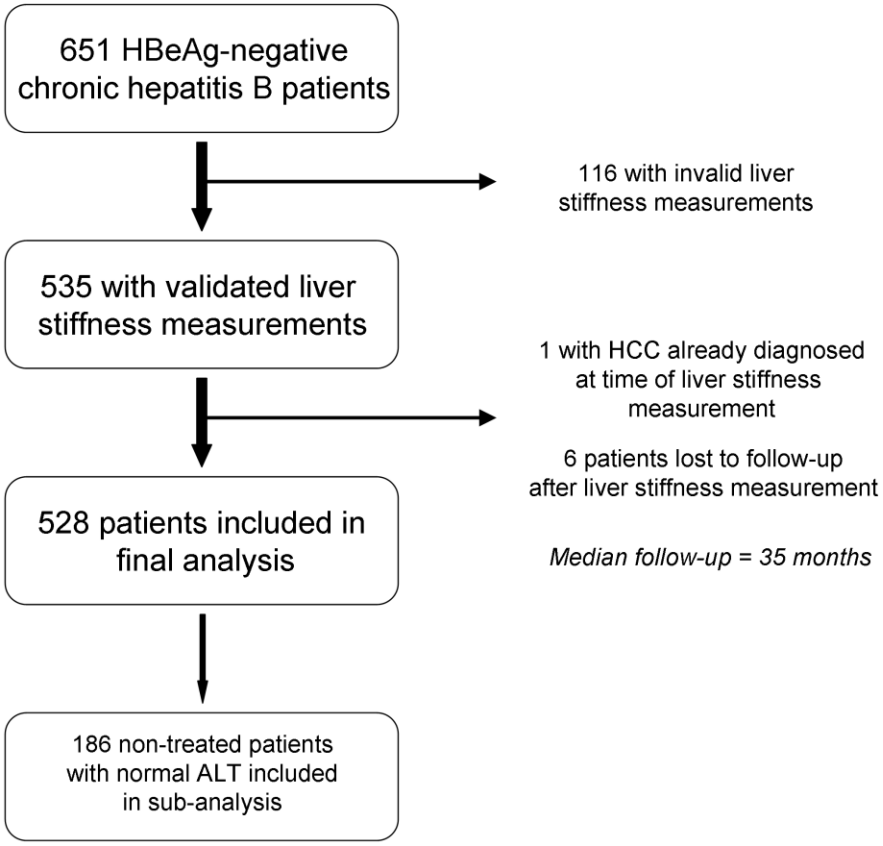

Figure 9.1 Patient recruitment algorithm

The patients were divided into 2 groups according to their liver stiffness measurements: less than 10 kPa and those with 10 kPa or greater (group 1 and 2 respectively). The cut-off value of 10 kPa was used as a recent study in chronic hepatitis C showed a significantly higher risk of HCC with liver stiffness above this threshold (Masuzaki et al., 2009). The demographic data and laboratory results of groups 1 and 2 are summarized in table 9.2. Patients with liver stiffness ≥10 kPa were older, had higher bilirubin, ALP, AST, ALT, GGT, globulin, AFP, log HBV DNA, and were more likely to be males and receiving antiviral treatment, compared to those patients with liver stiffness <10 kPa.

Table 9.1 Patient demographic data and baseline laboratory results

Parameter	Value
Number of patients	528
Age (years)	42 (18-86)
Male gender	324 (61%)
Bilirubin (umol/L)	11 (3-57)
ALP (U/L)	65 (23-208)
GGT (U/L)	23 (8-370)
AST (U/L)	24 (9-323)
ALT (U/L)	26 (8-487)
Albumin (g/L)	44 (30-51)
AFP (ng/mL)	3 (2-97)
Log HBV DNA (copies/mL)	4.0 (1.8-8.8)
Liver stiffness (kPa)	6.0 (1.5-56.1)
Treatment-naive	422 (80%)

* Continuous variables are expressed as median values (range). HCC = hepatocellular carcinoma, ALP = alkaline phosphatase, GGT = gamma-glutamyltransferase , AST = aspartate aminotransferase, ALT = alanine aminotransferase, AFP = alpha-fetoprotein, kPa = kilopascals.

Table 9.2 Demographic and laboratory data in patients with liver stiffness measurements of less than 10 kPa or over

Parameters	LSM <10 kPa Group 1 (n=445)	LSM ≥ 10 kPa Group 2 (n=83)	p value
Age (years)	41 (18-78)	49 (20-86)	<0.001
Male gender	57%	83%	<0.001
Bilirubin (umol/L)	10 (3-38)	13 (3-57)	0.003
ALP (U/L)	63 (23-154)	77 (44-208)	<0.001
GGT (U/L)	21 (8-261)	41 (11-370)	<0.001
AST (U/L)	22 (9-323)	34 (16-282)	<0.001
ALT (U/L)	25 (8-311)	43 (10-487)	<0.001
Albumin (g/L)	44 (30-51)	43 (35-50)	0.064
Globulin (g/L)	33 (24-46)	35 (26-44)	<0.001
AFP (ng/mL)	3 (2-47)	4 (2-97)	<0.001
Log HBV DNA (copies/mL)	3.9 (1.8-8.8)	4.9 (1.8-8.8)	<0.001
Liver stiffness (kPa)	5.6 (1.5-9.9)	12.6 (10.1-56.1)	<0.001
Treated patients	18%	30%	0.015

* Continuous variables are expressed as median values (range). ALP = alkaline phosphatase, GGT = gamma-glutamyltransferase , AST = aspartate aminotransferase, ALT = alanine aminotransferase, AFP = alpha-fetoprotein, kPa=kilopascals, LSM = liver stiffness measurement.

Hepatocellular carcinoma

Of the 528 patients, seven patients developed HCC during the follow-up period. Over a median follow-up of 35 months, there was a significantly higher cumulative incidence of HCC development in group 2 compared to group 1, as shown in figure 9.2. The cumulative incidence of HCC at 3 years in group 2 was 9%, compared with 0% in group 1 ($p<0.001$). Of the 445 patients who had liver stiffness measurement of less than 10 kPa, none had developed HCC during the follow-up period. Although there were significant differences with regards to age, gender, bilirubin, ALP, AST, ALT, GGT, globulin, AFP, log HBV DNA, and treatment status between the 2 groups, after multivariate analysis using Cox regression analysis, liver stiffness remained the only significant factor associated with subsequent development of HCC ($p=0.001$).

Two patients out of the 7 patients with HCC died during the follow-up period. A further patient died of decompensated liver cirrhosis. The cumulative liver-related mortality rate was significantly higher in group 2 compared to group 1 (4% vs 0% respectively, $p<0.001$) after 3 years of follow-up. None of the patients with liver stiffness measurement of <10 kPa died during the follow-up period. The cumulative liver-related mortality rate of these groups is shown in figure 9.3. After multivariate analysis by Cox regression analysis, only the liver stiffness measurements were shown to be significantly associated with subsequent mortality ($p=0.016$).

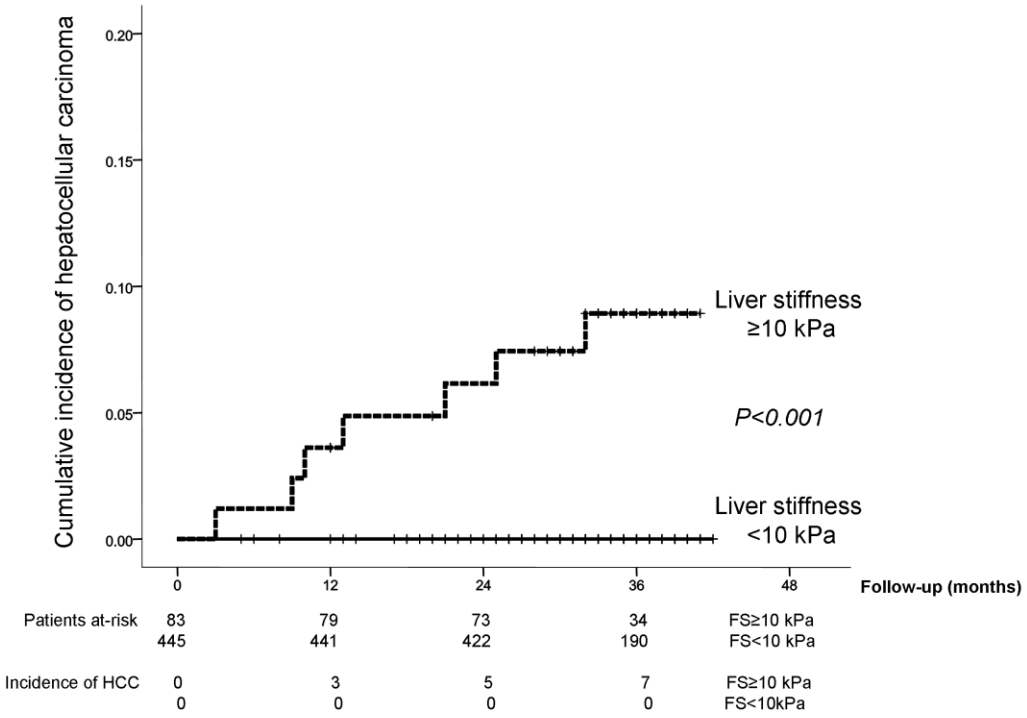

Figure 9.2 Cumulative incidence of hepatocellular carcinoma in patients with liver stiffness measurements <10 kPa and ≥10 kPa

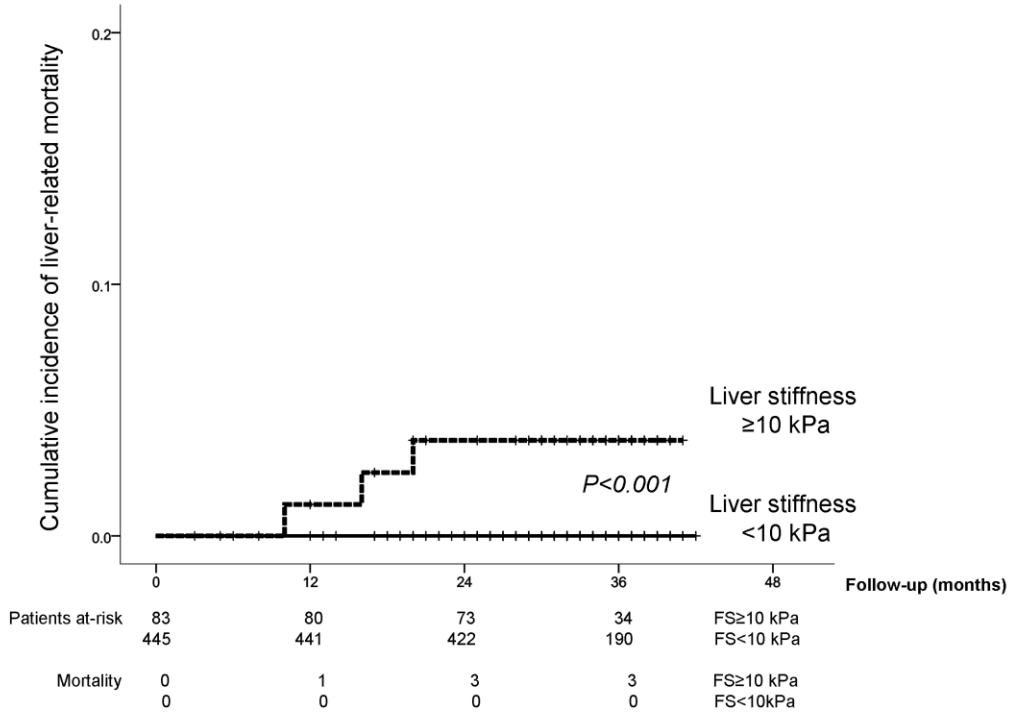

Figure 9.3 Cumulative liver-related mortality rates in patients with liver stiffness measurements <10 kPa and ≥10 kPa

Hepatitis flares

Of the 528 patients, 186 (35%) patients had normal ALT at the time of liver stiffness measurement, and also during the preceding 12 months. The normal cut-off values for ALT were defined as 30 U/L for males and 19 U/L in females in accordance with the new AASLD guidelines (Lok & McMahon, 2009). None of these patients were receiving antiviral therapy at the time of liver stiffness measurement. These patients were analyzed for their risk of developing subsequent flares of hepatitis (as defined by ALT >2x upper limit of normal). The total cumulative incidence of hepatitis flares in these patients was 16% at 3 years. There was a significantly higher cumulative incidence of hepatitis flares at 3 years in patients with liver stiffness measurements of ≥10 kPa compared with those with liver stiffness measurements of <10 kPa (46% vs 14% respectively, p=0.001), as shown in figure 9.4. Higher liver stiffness measurements and levels of AST were significantly associated with subsequent flares (table 9.3).

Table 9.3 Demographic and laboratory data of patients with normal ALT with subsequent flares

Parameters	Flares (n=27)	No flares (n=159)	P value
Age (years)	45 (25-66)	39 (20-78)	0.076
Bilirubin (umol/L)	10 (5-28)	11 (3-32)	0.418
ALP (U/L)	58 (36-76)	60 (23-116)	0.264
AST (U/L)	22 (15-43)	19 (9-62)	0.002
ALT (U/L)	19 (11-29)	18 (8-30)	0.159
GGT (U/L)	17 (8-47)	18 (8-123)	0.721
Albumin (g/L)	44 (40-49)	44 (30-51)	0.680
Log HBV DNA (copies/mL)	4.3 (1.8-6.7)	3.8 (1.8-8.8)	0.093
Liver stiffness (kPa)	6.9 (3.3-20.3)	5.2 (2.8-23.1)	<0.001

* Continuous variables expressed as median values

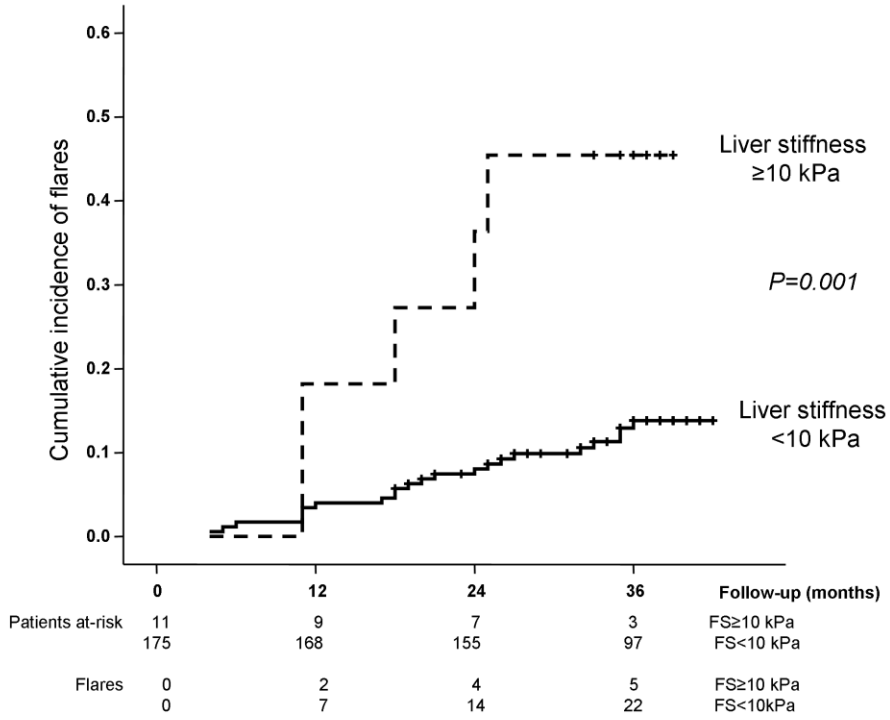

Figure 9.4 Cumulative rates of biochemical flare in patients with normal ALT and liver stiffness measurements <10 kPa versus ≥10 kPa

Discussion

There is relatively limited data on the use of transient elastography for monitoring treatment response and for long-term prognostic value. In a large cohort of 866 chronic hepatitis C patients from Japan followed for a mean duration of 3 years, those patients with higher liver stiffness measurements were shown to be at a significantly higher risk for HCC development (Masuzaki et al., 2009). The mean liver stiffness of this group was 11.9 kPa, suggesting a high prevalence of at-least severe fibrosis or early cirrhosis in their cohort. This is also reflected by the high rate of HCC within 3 years of follow-up, with 77 patients developing HCC. As HCC associated with chronic hepatitis C occurs almost exclusively in patients with established cirrhosis, only 2 patients developed HCC with liver stiffness measurements of <10 kPa. However, the study was able to demonstrate the increasing risk of HCC development with higher levels of liver stiffness values in cirrhotic patients, emphasizing the importance of further risk stratification of cirrhotic patients in chronic hepatitis C.

The current study focused on HBeAg-negative CHB. Although HBeAg negativity is often viewed favorably, the majority of HCC (approximately 75%) occurs in HBeAg-negative subjects (Fung, Yuen, Yuen, Wong, & Lai, 2007; Yuen et al., 2005). The median liver stiffness in our study population was 6.0 kPa, with the median liver biochemistry all within the normal range, followed up for a median of 35 months. The incidence of HCC in our study population is low. This is likely due to a combination of factors. Firstly, the median age of the study population was only 42 years, while the median age of development of HCC in our population has been shown to be over 55 years according to a previous study (Yuen et al., 2005). Secondly, the median liver

stiffness measurement was also lower than that described by the Japanese study (Masuzaki et al., 2009). Finally, a follow-up period of 3 years is also relatively short in the natural history of CHB disease.

Despite these factors which may reduce the power of the study to detect possible differences between the two groups of relatively younger aged patients, we were still able to show that in patients with liver stiffness measurement ≥10 kPa, there was a significant higher rate of HCC development and mortality. Because of the low incidence of HCC in our cohort, further risk stratification according to different increments of liver stiffness was not possible. However, we were able to identify a high risk group of patients in which regular surveillance with ultrasound in addition to AFP monitoring should be strictly implemented. This is an important consideration because earlier detection of HCC significantly increases chances of receiving curative therapy and improves long-term survival (McMahon et al., 2000; Yuen et al., 2000; B. H. Zhang et al., 2004). It has been suggested that CHB patients age >40 years should receive regular ultrasounds screening (Lok & McMahon, 2009). However, this had not been adopted by many countries, especially in regions where CHB is endemic, primarily due to the high cost involved. Since the median age (42 years) in the present study population was incidentally similar to this recommended age for screening, it may be possible to further streamline the criteria for screening for HCC by incorporating the measurement of liver stiffness in patients older than 40 years old. This may enhance the cost-effectiveness of a screening program, and further studies are highly recommended to confirm this.

In the multivariate analysis, age, gender, and viral load were not independent factors associated with HCC development. This is likely due to the relatively short duration of follow-up (3 years), and that in the short- to medium- term, liver stiffness maybe the more important prognostic indicator. Although this study showed that in patients with liver stiffness measurements of <10 kPa were at a lower risk, with no patients developing HCC, longer-term follow-up of these patients will be required to truly determine whether this group is at a negligible risk of long-term complications.

As part of the natural history of CHB, in patients who undergo loss of HBeAg, a significant proportion of these patients may still undergo hepatitis flares, requiring long term antiviral therapy (Hadziyannis & Vassilopoulos, 2001; Lai & Yuen, 2007). In untreated patients with normal ALT for at least 12 months, there was a significantly higher cumulative incidence of subsequent flares in those with liver stiffness measurements of ≥10 kPa compared to those with liver stiffness <10 kPa (46% vs 14% respectively, p=0.001).The use of transient elastography may identify those patients with normal ALT who are at a higher risk of subsequently developing biochemical flares, and therefore merit closer follow-up.

There are several limitations to the current study. Firstly, the incidence of non-alcoholic fatty liver disease is not known, and this may influence the liver stiffness measurements and liver disease outcome. However, it appears that although hepatic steatosis is common in CHB, it is not associated with viral factors and does not affect the severity of the liver disease (Shi et al., 2008). Secondly, not all patients had ultrasound screening performed at regular intervals, and at the time

of last follow-up, a small number of patients may have undetected and asymptomatic HCC. However, this is likely to occur in patients with liver stiffness measurements of <10 kPa and ≥10 kPa, and therefore should not affect the final comparison between these 2 groups. Thirdly, this study employed liver stiffness measurement at a single time point. Given that the majority of the subjects were non-cirrhotic, the reproducibility of transient elastography may have been compromised (Fraquelli et al., 2007). Serial or repeated liver stiffness measurements are likely to be more accurate.

In conclusion, transient elastography is not only useful in the assessment of liver fibrosis, but also has prognostic significance in patients with chronic viral hepatitis for complications including HCC. In HBeAg-negative CHB patients, liver stiffness measurement of ≥10 kPa was associated with a significantly increased risk of subsequent HCC development and mortality.

Chapter X

LONGITUDINAL FOLLOW-UP OF LIVER STIFFNESS MEASUREMENT IN CHRONIC HEPATITIS B – A 3-YEAR PROSPECTIVE STUDY

Introduction

In the preceding chapter we demonstrated an important application of transient elastography in the long-term prognosis of patients with CHB. The other important long-term application of liver stiffness measurement would be in monitoring disease progression and in assessing treatment response. In these 2 setting, paired liver biopsies are rarely employed.

In a recent study of patients with chronic hepatitis C, those patients that achieved sustained virological response (SVR) with antiviral therapy had a significant decline in liver stiffness values compared to those patients who did not achieve SVR (Ogawa et al., 2009). This finding suggests that liver stiffness measurement may be useful in assessing treatment response, and may signify regression of liver fibrosis with successful antiviral therapy.

For CHB, the longitudinal changes in liver stiffness values in the natural history and also in treated patients are currently not known. The aim of the current prospective study is to determine the longitudinal changes in liver stiffness long-term in both treated and untreated patients with CHB.

Patients and Methods

Four hundred and twenty six CHB patients seen at the Hepatitis and Liver clinics at Queen Mary Hospital, Hong Kong, with valid liver stiffness measurements performed in 2006 were included. These patients were followed-up regularly at 3-6 monthly intervals with routine hepatitis serology and liver biochemistry. A repeat liver stiffness measurement was performed at 3 years after the first measurement. Patients co-infected with HCV, HIV, or with other co-existing liver diseases including autoimmune hepatitis, alcoholic liver disease, Wilson's disease or PBC were not considered for the study. All patients were positive for HBsAg for at least 6 months before the diagnosis of CHB was made. This study was approved by the Institutional Review Board of the University of Hong Kong,

From the time of the initial liver stiffness measurement, all routine liver biochemistry and virological status were prospectively recorded until the time of follow-up liver stiffness measurement. Patients were grouped according to their virological status for analysis into HBeAg-positive, HBeAg-negative, and HBsAg-loss group. Those patients in the HBsAg-loss group had known CHB with subsequent loss of HBsAg during follow-up prior to the initial liver stiffness measurement. Levels of HBV DNA were measured using the Cobas Taqman assay (Roche Diagnostics, Branchburg, NJ), with a lower limit of detection of 60 copies/mL.

For liver stiffness measurements, at least 10 valid measurements were obtained for each patient. The results were included in the final analysis only if both the success rate was greater than 50%

and the IQR-to-liver stiffness ratio was less than 0.30. Those patients with liver stiffness measurements outside these criteria were not considered for the study. The median values of the validated measurements were representative of the liver stiffness and expressed in units of kPa. Informed consent was obtained from each patient.

Statistical analysis

All statistical analyses were performed using SPSS version 17.0 (SPSS Inc, Chicago, IL). Continuous variables with skewed distribution were analyzed using Mann-Whitney test. Paired-related continuous variables were analyzed using the Wilcoxon paired test. Chi-squared test was used for categorical variables, and Fisher's exact test when appropriate. Multivariate analysis was performed using bivariate logistic regression on variables found to be significant on univariate analysis. The normal cut-off values used for ALT in males and females were 30 and 19 U/L respectively, in accordance with the current AASLD treatment guidelines (Lok & McMahon, 2009). A p-value of <0.05 was considered statistically significant.

Results

A total of 426 patients were included. All patients had valid liver stiffness measurement performed using transient elastography between January 2006 and December 2006. Liver stiffness measurement was repeated with a median time interval of 38 months (range, 30-44). Of the 426 patients, 38 (9%) were HBeAg-positive, 293 (69%) were HBeAg-negative, and 95 (22%) were patients who had loss of HBsAg prior to the initial liver stiffness measurement. The patient characteristics and liver stiffness values are summarized in table 10.1. At the initial time point, HBeAg-positive patients had a significantly higher liver stiffness values compared with HBeAg-negative patients (6.7 vs 6.1 kPa respectively, p=0.049) and HBsAg-loss patients (6.7 vs 5.6 kPa respectively, p<0.001). HBeAg-negative patients had a significantly higher liver stiffness values compared with HBsAg-loss patients (6.1 vs 5.6 kPa respectively, p=0.006). At the second time point, there was no significant difference observed between HBeAg-positive vs HBeAg-negative patients (6.2 vs 5.7 kPa respectively, p=0.120), and HBeAg-negative vs HBsAg-loss patients (5.7 vs 5.4 kPa respectively, p=0.355), although a significantly higher liver stiffness values was observed in HBeAg-positive patients compared with HBsAg-loss patients (6.2 vs 5.4 kPa respectively, p-0.034) (Figure 10.1).

There was a significantly lower liver stiffness values in the follow-up measurement (5.6 kPa, range, 3.0-46.4) compared to the initial measurement (6.0 kPa, range, 1.5-28.0) in the overall study group (p<0.001). However, on further subgroup analysis, this significant decline was only observed in the HBeAg-negative patients, and no significant changes were observed in the HBeAg-positive patients or HBeAg-loss patients (table10.2).

Table 10.1 Patient characteristics and laboratory results

Parameters	Results
Total number of patients (n)	426
Age (years)	44 (19-81)
Sex (male)	276 (65%)
Virology	
HBeAg-positive	38 (9%)
HBeAg-negative	293 (69%)
HBsAg-loss	95 (22%)
Laboratory results at entry	
Bilirubin (umol/L)	11 (2-40)
ALP (U/L)	65 (27-153)
GGT (U/L)	23 (8-261)
AST (U/L)	23 (9-125)
ALT (U/L)	25 (8-273)
Log HBV DNA (copies/mL)	3.7 (1.8-8.8)
Antiviral therapy	110 (26%)

Continuous variables are expressed as median values (range)

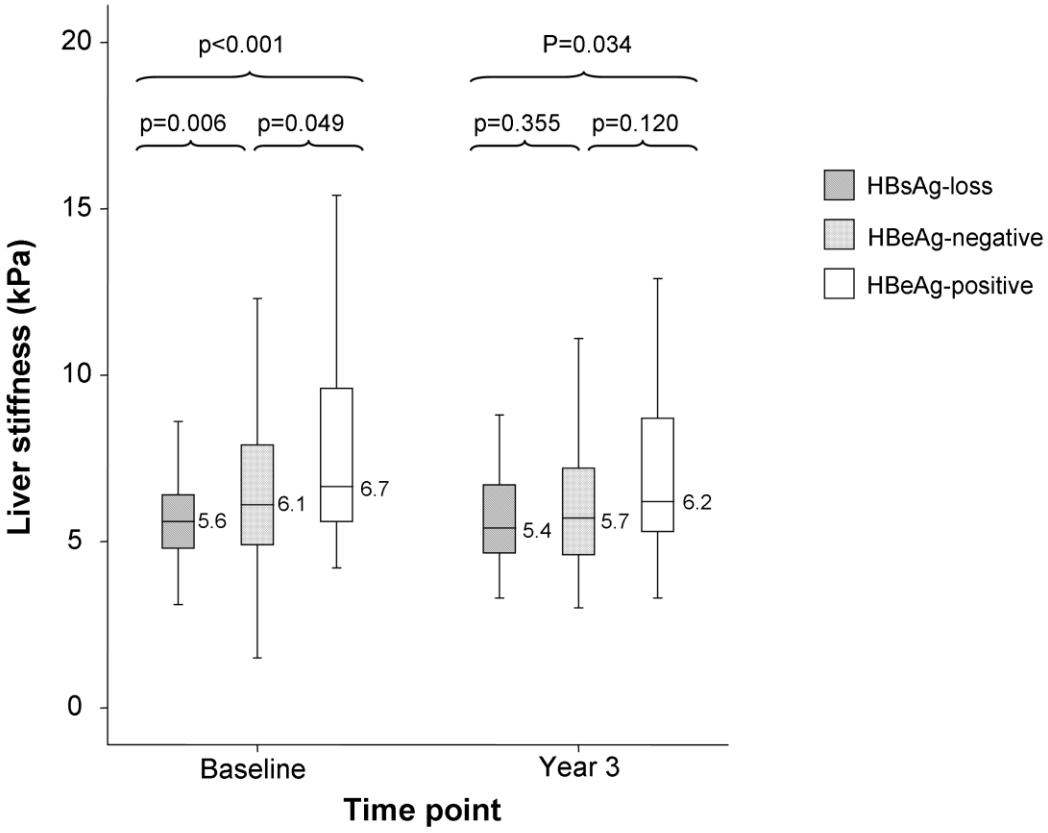

Figure 10.1 Median liver stiffness measurements at baseline and at year 3

Patients were subsequently divided into 4 different groups according to their ALT levels. Group 1 patients had normal ALT at the initial scan and elevated ALT at the time of repeat liver stiffness measurement. Group 2 patients had an elevated ALT at the initial liver stiffness measurement, with subsequent normalization of the ALT at the follow-up liver stiffness measurement. Group 3 patients had normal ALT levels at both time points, whereas patients in group 4 had elevated ALT levels at both time points.

Table 10.2 Liver stiffness measurements at the time of study entry and at follow-up

Parameters	Initial	3 year follow-up	p-value
Total patient (n=426)			
Liver stiffness (kPa)	6.0 (1.5-28.0)	5.6 (3.0-46.4)	<0.001
HBeAg-positive patients (n=38)			
Liver stiffness (kPa)	6.7 (4.2-15.4)	6.2 (3.3-16.6)	0.078
HBeAg-negative patients (n=293)			
Liver stiffness (kPa)	6.1 (1.5-28.0)	5.7 (3.0-46.4)	<0.001
HBsAg-loss patients (n=95)			
Liver stiffness (kPa)	5.6 (3.1-12.4)	5.4 (3.3-12.2)	0.902

Continuous variables are expressed as median values (range)

Treated group

Of the 426 patients, 110 (26%) received oral antiviral therapy, and 316 (74%) were not on antiviral therapy. Patients who received antiviral therapy were treated with the following agents: lamivudine (n=49), entecavir (n=41), adefovir (n=6), tenofovir (n=3), telbivudine (n=1), and combination therapy (n=10). Twenty three patients were HBeAg-positive and 87 patients were HBeAg-negative. There was a significantly lower liver stiffness values at 3 years compared with the liver stiffness values at study entry (6.1 vs 7.3 kPa respectively, $p<0.001$). However, after stratifying the patients into the different ALT groups, the significant decline was only observed in the group of patients who had elevated ALT at study entry, with normalization of ALT after 3 years (group 2). There were no significant changes in liver stiffness values between these two time points for patients in groups 1, 3, and 4 (figure 10.2).

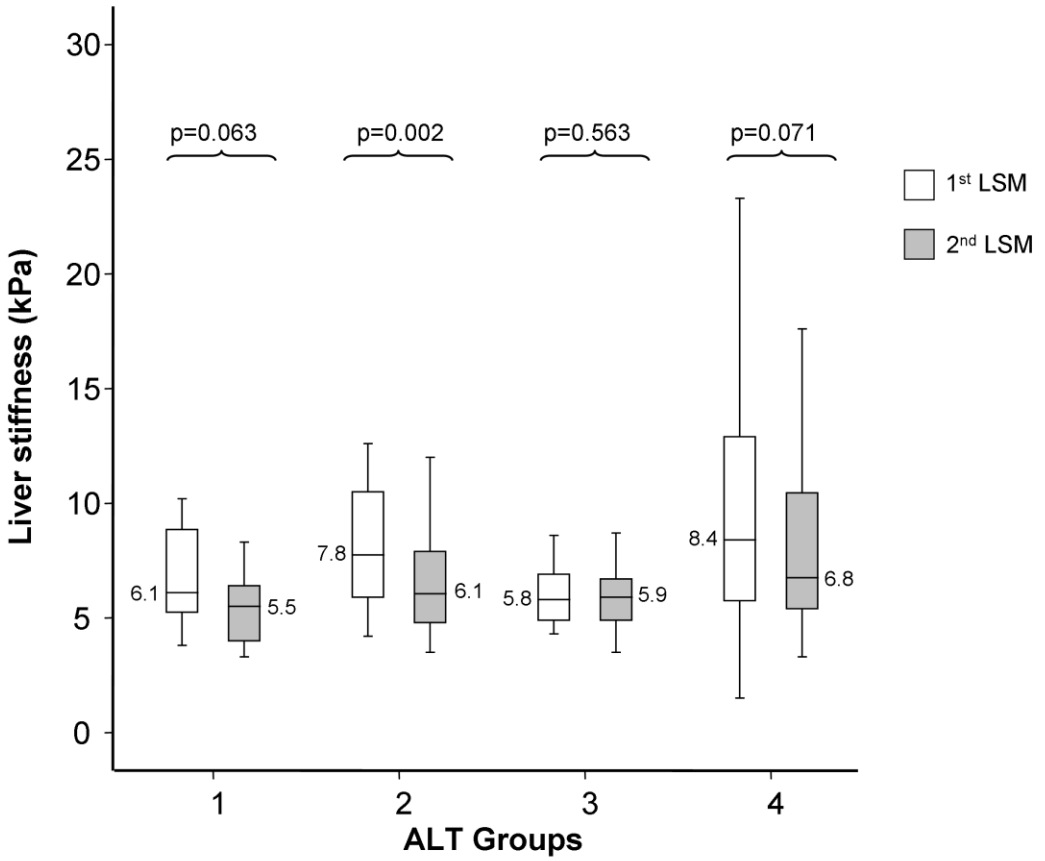

Figure 10.2 Differences in liver stiffness in different ALT groups between the two time-points in treated patients

Non-treated group

The remaining 316 (74%) patients did not receive antiviral therapy during the study period. Of the non-treated patients, 221 were HBsAg-positive, and 95 were patients who had HBsAg-loss previously. There was a decline noted in the 221 patients, with a significantly lower liver stiffness values at the follow-up time point compared with the initial liver stiffness values (5.4 vs 5.9 kPa, p=0.018). After stratification into the different ALT groups, only those patients who were in group 3 (normal ALT at both study entry and at the 3 year follow-up) had a significantly lower liver stiffness values at follow-up compared to at the time of study entry (4.9 vs 5.3 kPa respectively, p=0.005). There was no significant difference observed in liver stiffness values between the 2 time points in groups 1, 2, and 4 (figure 10.3).

In those patients with prior loss of HBsAg, there was overall no significant difference observed in liver stiffness values between the 1st and 2nd time points (5.6 vs 5.4 kPa respectively, p=0.902). However, after stratifying the patients into different ALT groups, a significant decline was noted in group 3 patients (normal ALT at both study entry and at the 3 year follow-up) with a higher liver stiffness values noted at study entry compared to the time of follow-up (5.4 vs 5.1 kPa respectively, p=0.026). In group 4 patients (those with elevated ALT at study entry and at follow-up), there was a significantly higher liver stiffness values at the time of follow-up compared to study entry (6.5 vs 6.1 kPa respectively, p=0.045). No significant difference was seen in groups 1 and 2 (figure 10.4).

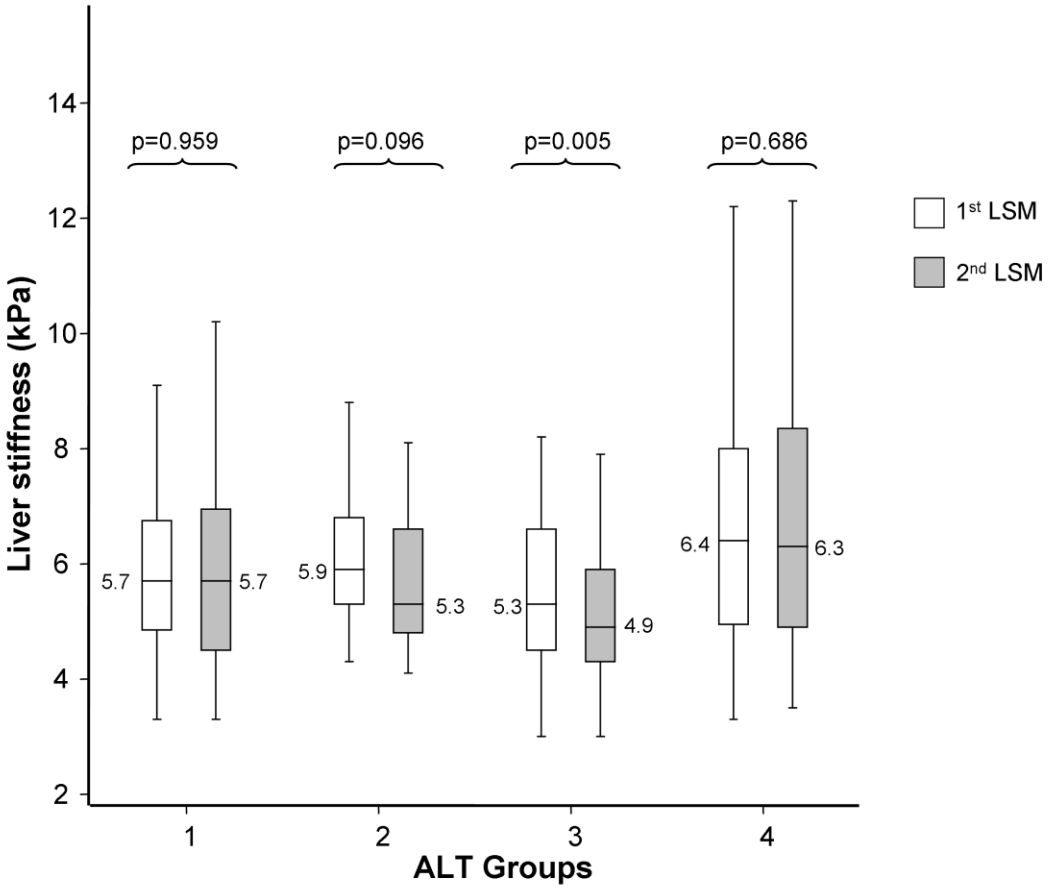

Figure 10.3 Differences in liver stiffness in different ALT groups between the two time-points in non-treated patients

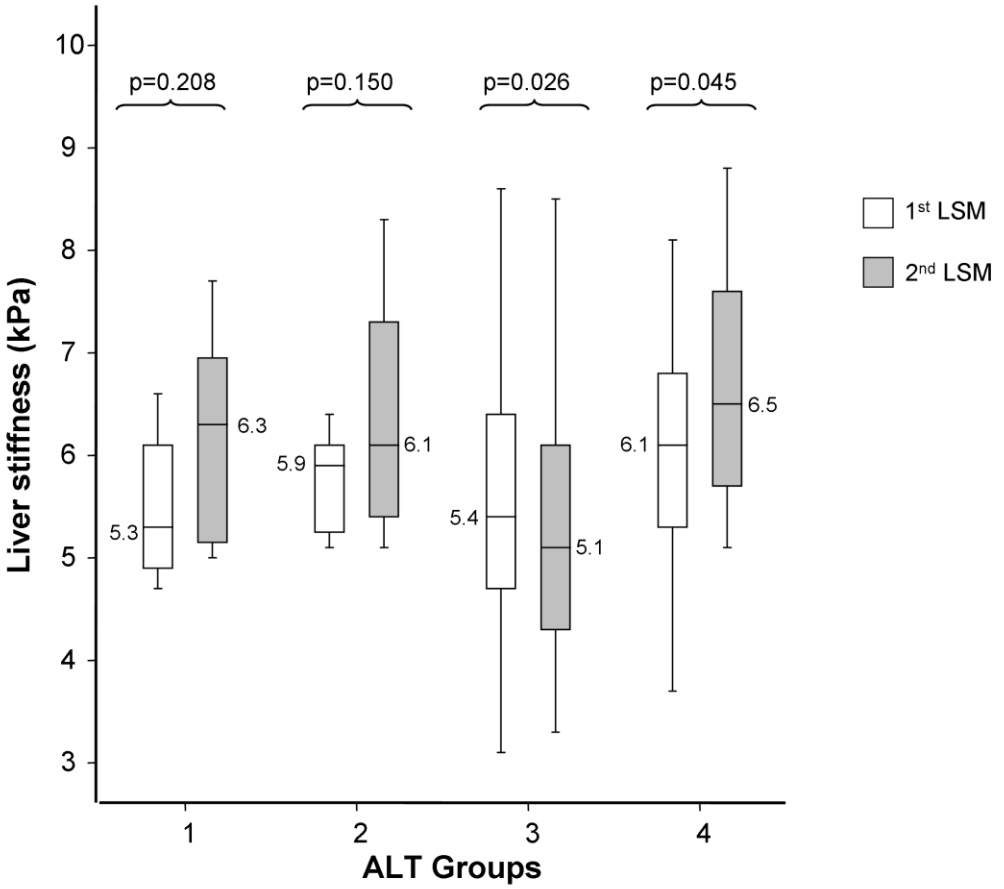

Figure 10.4 Differences in liver stiffness in different ALT groups between the two time-points in patients with HBsAg loss

Decline in liver stiffness measurements

To identify factors associated with a significant decline in liver stiffness values for HBsAg-positive patients, they were divided into those who achieved ≥1 kPa decline on the follow-up scan and those who did not. Those factors associated with a significant decline in liver stiffness include a higher AST and ALT level at initial measurement, lower ALT at follow-up measurement, and antiviral treatment. There was also a trend for lower AST, albumin and AFP at the time of follow-up in patients with a significant drop in liver stiffness compared with those that did not (table 10.3). After multivariate analysis by logistic regression, only antiviral therapy (p=0.011) and the follow-up ALT (p=0.024) were significantly associated with ≥1 kPa decline.

Table 10.3 Factors associated with significant decline (≥1 kPa) in liver stiffness measurements after 3 years of follow-up

Parameter	<1 kPa drop	≥1 kPa drop	P value
Antiviral therapy	25.5%	44.4%	<0.001
Study entry			
AST (U/L)	23 (9-125)	27 (12-104)	0.002
ALT (U/L)	26 (8-257)	31 (10-273)	0.045
Follow-up (3 years)			
ALT (U/L)	29 (9-336)	26 (7-307)	0.013

Continuous variables are expressed as median values (range)

Discussion

The general non-acceptance and safety concerns with liver biopsies have precluded its use in large population screening studies of liver fibrosis and cirrhosis, and in longitudinal studies in which repeated determinations are required. Liver stiffness measurement by transient elastography has now become readily available in many parts of the world for assessing liver fibrosis non-invasively. With the ease, safety, and rapidity of performing transient elastography, the potential applications for liver stiffness measurement extends beyond liver fibrosis assessment alone. Other possible uses include the long-term monitoring of patients with chronic liver disease, and in the assessment of treatment response in these patients. There is currently very little data on long term monitoring of liver stiffness in patients with chronic liver disease. Several recent studies have shown that interferon treatment reduced liver stiffness in patients with chronic hepatitis C, especially in those patients who achieved SVR (Arima et al.; Ogawa et al., 2009; J. H. Wang et al., 2010). In hepatitis B, our longitudinal 1-year follow-up study of patients with acute severe flares revealed a significant decline in liver stiffness values with normalization of liver transaminases (Fung et al., 2008). However, the liver stiffness values at baseline were shown to be spuriously high in the setting of severe acute hepatitis, and not reflective of the underlying fibrosis stage. Therefore, the change in liver stiffness values in this setting is unlikely to be attributed to reversal of fibrosis.

The current study is the first prospective study reporting on the longitudinal changes in liver stiffness using transient elastography in patients with CHB over a median period of more than 3 years. There was a significant decline in liver stiffness values between the two time points in the

overall study population, and in the treated and non-treated subgroups. Within the treated subgroup, the significant decline was only observed in patients who had a subsequent normalization of their ALT. For non-treated patients, the significant decline was observed only in the patient group where the ALT remained normal at both time points. In the HBsAg-loss group, there was a significant decline in liver stiffness values for those patients with normal ALT at both time points, in contrast to those with elevated ALT at both time points, whereby there was a significant increase in liver stiffness values. The most likely cause of ALT elevation in our population in this group who had HBsAg loss is fatty liver disease and herbal intake.

In HBsAg-positive patients, only antiviral therapy and the ALT at the second time point has been shown after multivariate analysis to be significant factors associated with a ≥1 kPa decline in liver stiffness values after 3 years of follow-up. This is consistent with the fact that antiviral therapy can reverse the stages of fibrosis, particularly with prolonged therapy (Dienstag et al., 2003; Hadziyannis et al., 2006; Liaw, Chang et al., 2008; Marcellin et al., 2008). However, histological confirmation will be required to establish this as the cause of the decline in liver stiffness. As described previously, a significant decline in liver stiffness values in those treated patients were seen only in the group of patients where their ALT decreased or were normalized at the time of follow-up measurement. Therefore, it is likely that a decline in ALT also plays a role in the decline in liver stiffness, indicating a potential role of inflammation in affecting liver stiffness. In previous studies, liver stiffness values have been shown to be significantly correlated with ALT levels, and even milder elevations of ALT were associated with significantly higher levels (Fung, Lai, Fong et al., 2008).

There are several limitations of the current study. Firstly, the number of HBeAg-positive patients with longitudinal liver stiffness measurements is small, therefore the results may not be conclusive for this group, and further stratification of this group was not possible for some of the analysis. Secondly, liver histology was not available in this study. The lack of histology highlights the important fact that liver biopsy is not readily accepted by patients because of the perceived risks, and the importance of non-invasive tests.

In conclusion, significant decline in liver stiffness occurs in CHB patients after 3 years of follow-up. However, this decline is only observed in specific groups of both treated and non-treated patients. Both antiviral treatment and follow-up ALT are significant factors associated with a decline of ≥1 kPa in liver stiffness.

Chapter XI

CLINICAL APPLICATION OF LIVER STIFFNESS MEASUREMENT IN LIVER DISEASE USING TRANSIENT ELASTOGRAPHY

Introduction

In the introductory chapters, the importances of determining liver fibrosis were emphasized. In patients with CHB, determination of the severity of liver fibrosis is important for prognostic reasons, and for identifying patients who will benefit from treatment. For those patients already receiving treatment, assessment of liver fibrosis can determine their response to treatment. In addition, hepatocellular carcinoma and variceal screening can be implemented for patients identified with underlying cirrhosis. Currently, liver biopsy remains the gold standard for assessing liver fibrosis, even though the diagnostic accuracy is limited by the specimen size and fragmentation, sampling error, and inter-observer variability. Furthermore, liver biopsy is an invasive procedure which can be associated with significant morbidity and rarely mortality, rendering it less acceptable by patients (Bravo et al., 2001). Clearly there is a need for an alternative method for assessing fibrosis which is more acceptable to patients without compromising diagnostic accuracy.

The exponential increase in the development of non-invasive methods to assess liver fibrosis has changed the approach to the way clinicians manage patients with CHB in the last few years. In Hong Kong alone, transient elastography has evolved from the research arena to become a widely available investigation for daily clinical use within the last 3 years. As shown in the previous chapters, transient elastography has wider applications that are not limited to the assessment of liver fibrosis. In this final chapter, the different applications of transient elastography in the clinical setting will be summarized together with the findings from the studies in the current thesis.

Assessment of fibrosis

The current primary indication for performing transient elastography is for the assessment of liver fibrosis to guide treatment decisions in a wide number of chronic liver diseases. Studies have been performed on a wide range of liver diseases including CHB, chronic hepatitis C, HCV/HIV co-infection, non-alcoholic steatohepatitis, PBC, primary sclerosing cholangitis (PSC), and recurrent hepatitis C after liver transplantation (Carrion et al., 2006; Corpechot et al., 2006; V de Ledinghen et al., 2006; Gomez-Dominguez et al., 2008; Marcellin et al., 2009; Yoneda et al., 2008), showing high accuracy for predicting fibrosis. Other conditions such as cystic fibrosis and recessive multicystic kidney diseases have shown a promising role for transient elastography (Kummer et al., 2011; Menten et al., 2010). However, as described in the following sections, the performance of transient elastography and the cut-off values obtained are dependent on the underlying disease conditions, and therefore validation studies are a prerequisite for different diseases.

Correlation of liver stiffness measurements with fibrosis

The most important aspect of liver stiffness measurement in clinical practice is whether the results can be accurately correlated with the different stages of fibrosis seen with liver histology. As shown previously, liver stiffness correlates well with the amount of fibrosis in the liver. One of the important aspects regarding the use of transient elastography is that there is a different cut-off value used for different stages of fibrosis. In addition, these individual cut-off values corresponding to the different fibrosis stages are disease-specific, with different values used for different conditions. These cut-off values have been derived from validation studies, and are

dependent also on the population of patients that were recruited in those studies. An example of cut-off values used for different stages of fibrosis for different diseases is shown in table 11.1.

Table 11.1 Different cut-off levels for different ranges of fibrosis for different diseases

Study	Population	F≥2	F≥3	F=4
Marcellin (2005)	CHB	7.2	8.1	11.0
Castera (2005)	CHC	7.1	9.5	12.5
De Ledinghen (2006)	HCV + HIV	-	-	14.5
Coco (2004)	CHB & CHC	8.3	-	14
Foucher (2005)	Chronic liver disease	7.2	12.5	17.6
Nguyen-Khac (2008)	Alcoholic liver disease	7.8	11	19.5
Corpechot (2006)	PBC & PSC	7.3	9.8	17.3

CHB = chronic hepatitis B; CHC = chronic hepatitis C; HCV + HIV = hepatitis C virus and human immunodeficiency virus co-infection; PBC = primary biliary cirrhosis; PSC = primary sclerosing cholangitis

Therefore, the results of the liver stiffness measurement should be interpreted according to the specific cut-offs used for the underlying disease. The reason for the difference in cut-off values is not known, although the distribution of fibrous material is dependent on the origin of liver injury,

which in turn is dependent on the underlying pathology. This may explain partially the differences.

Because of the variability in cut-off values (even within the same disease), the use of cut-off ranges rather than a single cut-off value should be employed. For example, in patients with liver stiffness <7.0 kPa, there is likely minimal or no fibrosis, whereas cirrhosis is likely in patients with liver stiffness >12.5 kPa (figure 11.1).

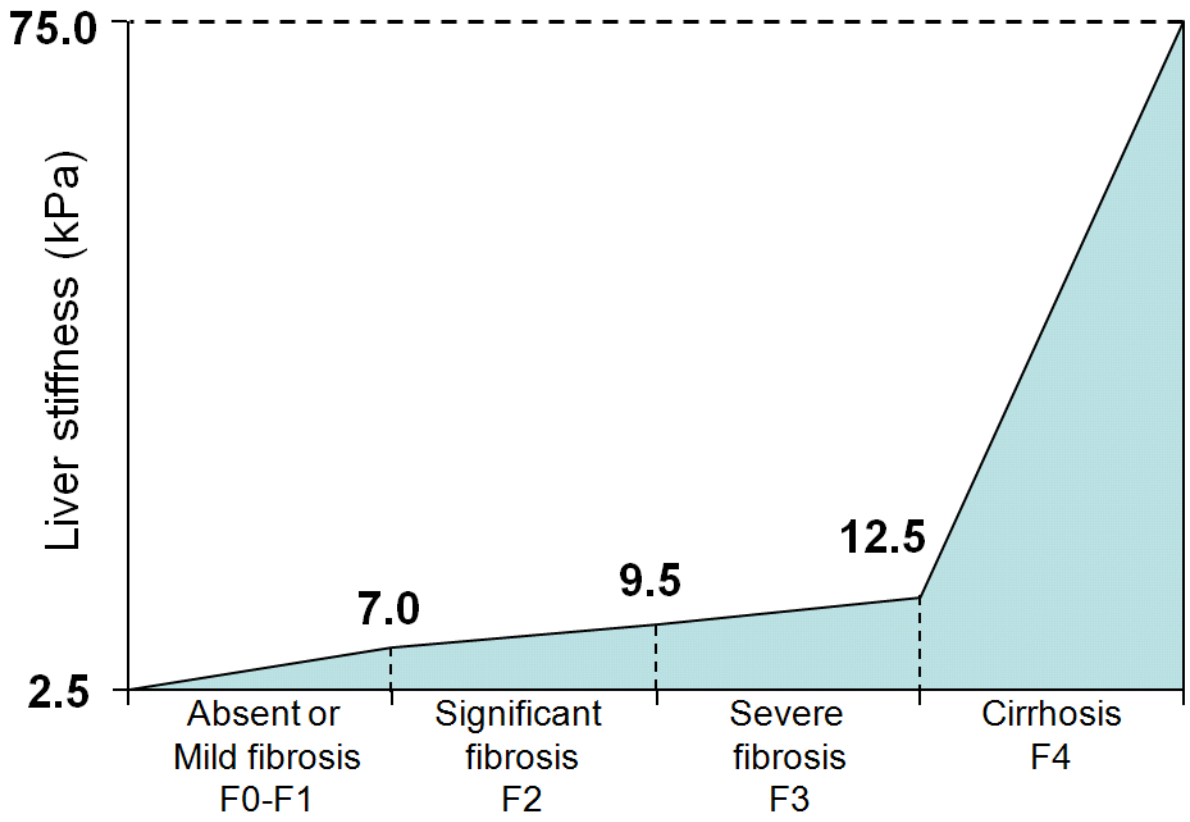

Fig 11.1 Examples of ranges of liver stiffness measurements for different stages of fibrosis

The consistent finding of the individual validation studies is the excellent performance of transient elastography for the diagnosis of severe fibrosis and cirrhosis. However, for lesser degree of fibrosis, the performance was more heterogeneous, and dependent on the underlying liver disease. In this group, the so-call "grey" area, a liver biopsy may still be recommended to confirm the stage of fibrosis prior to making a therapeutic decision. The use of transient elastography may decrease a significant number of liver biopsies. However, it is unlikely that complete avoidance will be achieved. Figure 11.2 gives an example of how treatment guidelines may incorporate transient elastography into their decision-making process.

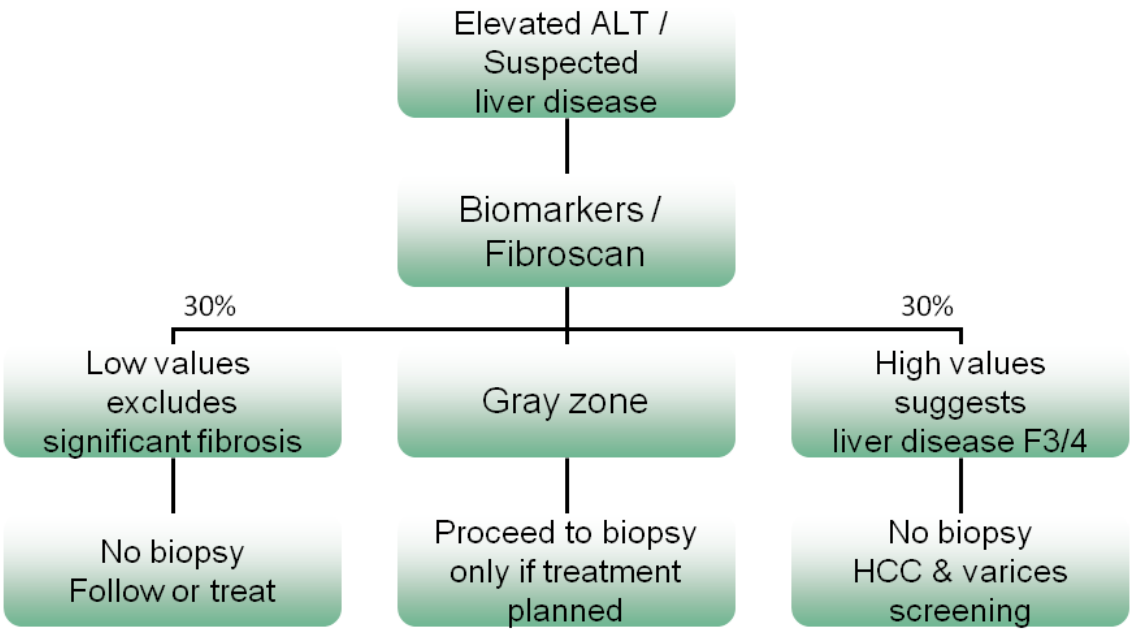

Figure 11.2. Proposed treatment algorithm incorporating the use of transient elastography in management decisions for chronic hepatitis C (Afdhal & Curry, 2007).

CHB is endemic in Hong Kong. In New Zealand, CHB is also endemic in certain ethnic groups, including the Maori and Polynesian people (Robinson, Bullen, Humphries, Hornell, & Moyes, 2005). In the current APASL guidelines for CHB treatment, liver biopsy is recommended for patients who are over the age of forty with ALT<2x ULN and HBV DNA >20,000 IU/mL (HBeAg-positive) or > 2,000 IU/mL (HBeAg-negative) (Liaw, Leung et al., 2008). The EASL guidelines recommend liver biopsy in those with elevated ALT and/or HBV DNA >2000 IU/mL. The AASLD guidelines recommend liver biopsies for those age over 40, with elevated ALT levels, and HBV DNA >2000 IU/mL (for HBeAg-negative patients). Those patients with significant fibrosis would be candidates for antiviral therapy. Those patients who would be recommended for liver biopsy according to the guidelines would be ideal candidates for transient elastography where biopsy can be avoided. But we must keep in mind that even mild to moderate levels of ALT elevation can also affect the liver stiffness measurements. Therefore it is likely that future diagnostic algorithm that incorporates the use of transient elastography as part of the initial liver assessment will need to take into account the effect of elevated ALT levels. An example of how different cut-off levels may be used with different ALT levels is shown in figure 11.3. In patients with liver stiffness values outside these criteria, liver biopsy should still be considered (Chan, Wong, Choi et al., 2009).

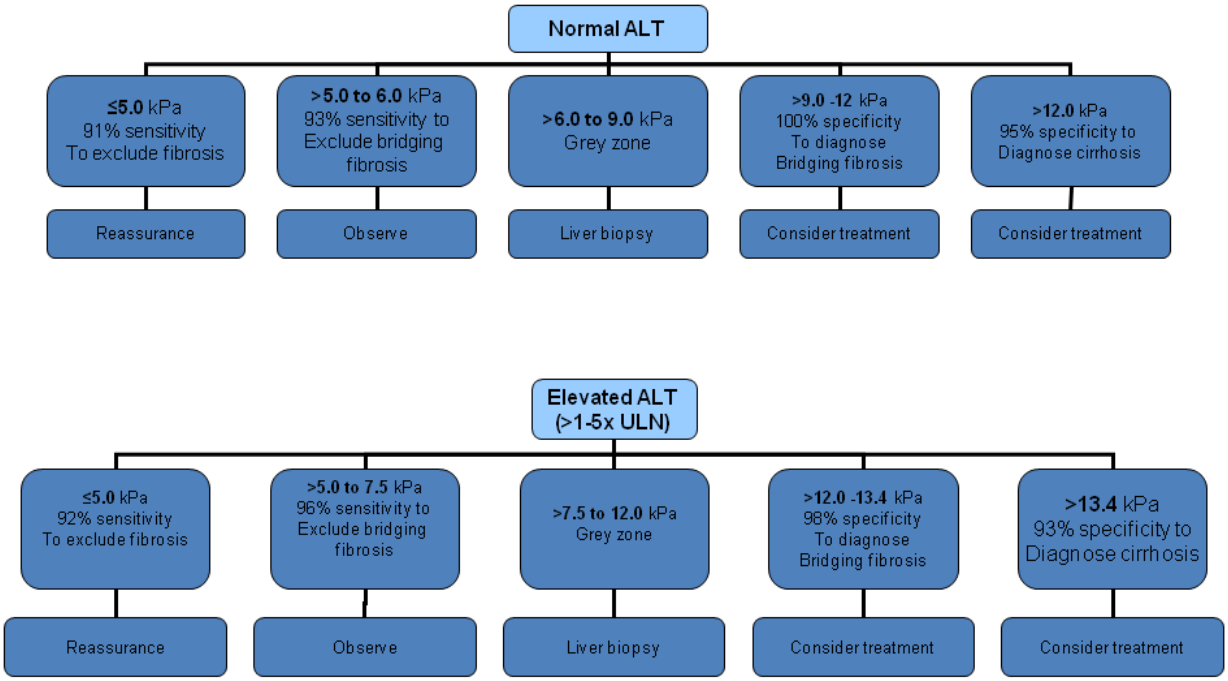

Figure 11.3 Proposed treatment for CHB with different cut-offs for different ALT levels (Chan, Wong, Choi et al., 2009).

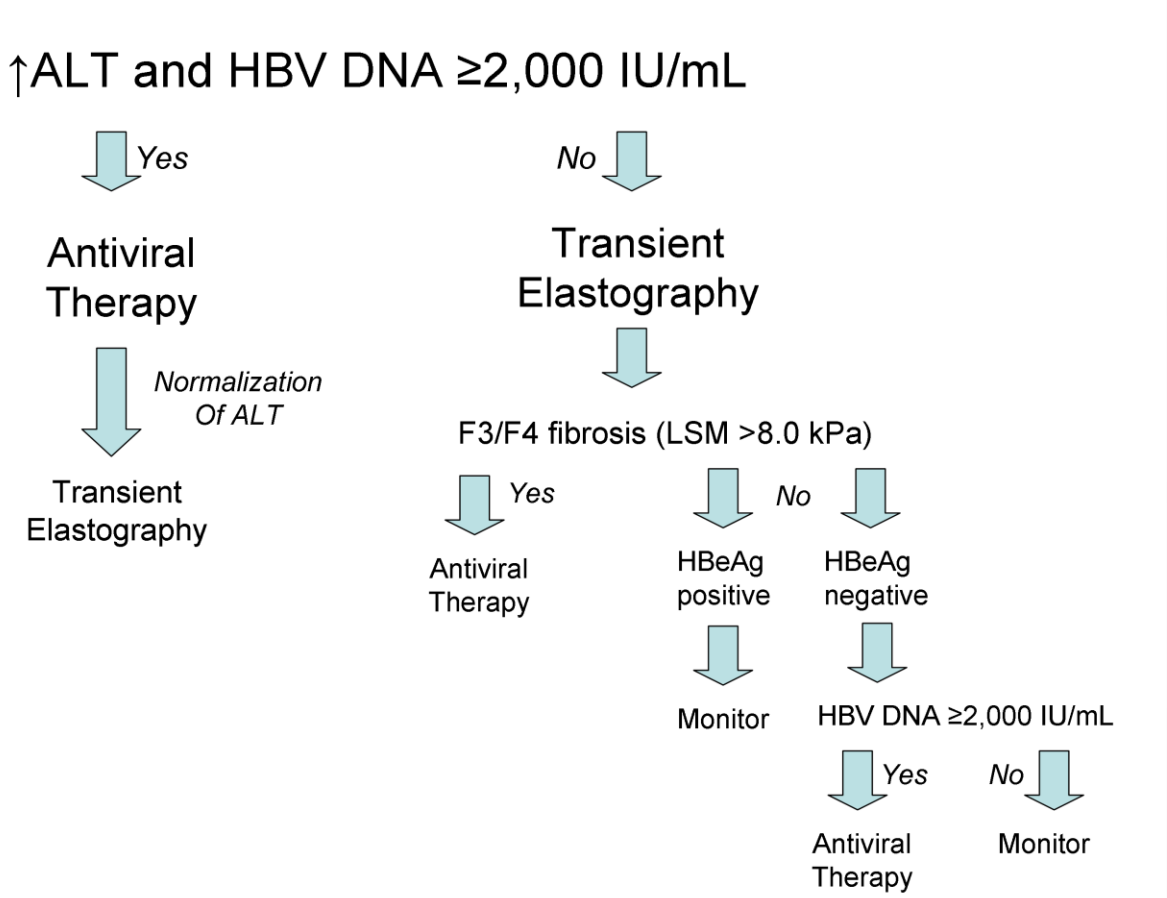

Figure 11.4 Proposed role of transient elastography treatment algorithm for CHB

In the era of highly potent oral antiviral agents with high genetic barrier to resistance, the risk of therapy to patients is minimal in the context of resistance development. The lowering of ALT and HBV DNA threshold for treatment observed in the last decade means more patients are eligible for treatment. Liver biopsy is most useful in patients who do not meet the clear cut guidelines. Although the accuracy of transient elastography is lowered in patients with elevated ALT, this may not be of great concern in the modern management of CHB where these patients are likely to be treated. In figure 11.4, a simplified proposal to incorporate transient elastography

into current treatment guidelines is shown. For those patients with elevated ALT and HBV DNA ≥2000 IU/mL, antiviral can be commenced, and transient elastography can be performed once the ALT is normalized. In those patients with evidence of cirrhosis, surveillance for complications of cirrhosis can be implemented. For patients with normal ALT levels, transient elastography can be performed and treatment can be started for those with F3 fibrosis/cirrhosis to prevent further disease progression or decompensation in the case of cirrhotic patients. In patients without evidence of advanced fibrosis or cirrhosis, then the HBeAg status can be used for treatment decision. For HBeAg-positive patients, they are likely in the immunotolerant phase and regular monitoring will suffice. For HBeAg-negative patients, treatment decisions will be dependent on the level of HBV DNA. In inactive carriers, the HBV DNA should remain very low.

Assessment of treatment response

On-treatment assessment of liver fibrosis has been used as a surrogate marker of treatment response and success in patients with chronic liver diseases. In CHB patients, long-term antiviral treatment has been shown to improve histological stages of fibrosis using paired liver biopsies (Dienstag et al., 2003; Hadziyannis et al., 2006). However, outside of clinical trial settings, on-treatment assessment using liver biopsy is usually not feasible. A non-invasive technique such a transient elastography is ideal in this clinical setting. In chronic hepatitis C patients treated with pegylated interferon alpha-2b and ribavirin, liver stiffness values were significantly decreased (compared to pre-treatment values) in those patients with SVR compared with those that did not achieve SVR (Ogawa et al., 2009). In our 3-year study of CHB patients, the decline in liver

stiffness with antiviral therapy was only seen in the group with elevated ALT at baseline with subsequent normalization of ALT at the follow-up scan. The preliminary studies so far suggest a role of liver stiffness measurement to assess improvements in fibrosis stages of patients on treatment. However, the decline in liver stiffness values may be due to decline in inflammation rather than fibrosis, and further studies with histological follow-up are required to confirm the improvement in fibrosis stages in this setting. This paradigm may also extend to other disease entity such as haemochromatosis, autoimmune hepatitis, and alcoholic steatohepatitis, whereby disease control may reduce the inflammation or "loading" of the liver, thus may reduce liver stiffness.

Disease prognosis

In patients with established cirrhosis, there is evidence that the degree of liver stiffness elevation may be predictive of underlying complications related to cirrhosis. Correlation between liver stiffness values and the presence of oesophageal varices has been reported in several studies (Bureau et al., 2008; Castera et al., 2009; Kazemi et al., 2006; Vizzutti et al., 2007). However, not all studies have shown correlation between liver stiffness values and variceal size (Vizzutti et al., 2007). In addition, the cut-off liver stiffness value for prediction of large (grade 2 or 3) varices in these studies was variable with suboptimal specificity. Without data from further validation studies, the use of transient elastography is currently insufficient to predict the presence or absence of oesophageal varices in cirrhotic patients, and upper endoscopy is still required for screening.

As transient elastography is a relative new technology, the long-term prognostic application of liver stiffness measurement is now only becoming available. In a large prospective study of over 800 patients with chronic hepatitis C followed up for a mean period of 3 years, liver stiffness was an independent predictor of subsequent development of HCC (Masuzaki et al., 2009). We have also described a prospective long-term cohort of patients with CHB showing a significant association between higher liver stiffness measurement and mortality and HCC development. These findings show that there is potential for transient elastography to be used as a screening tool to stratify patients' risk of HCC, and to implement screening and closer monitoring for high risk patients.

Screening

One of the major advantages of non-invasive investigations is their potential use as a screening tool. This is especially useful in populations where liver disease is prevalent. In our large population study of over 1,300 patients with CHB in Hong Kong, 34% of patients were found to have severe fibrosis. Even in patients with ALT 0.5-1 x ULN, 30% had severe fibrosis (Fung, Lai, But et al., 2008). Identifying asymptomatic patients with significant fibrosis and cirrhosis through screening will have significant implications on the management of this disease. Other potential population for screening includes those at risk of non-alcoholic fatty liver disease, and those with significant alcohol intake or a history of intravenous drug use. The ability to screen large number of subjects makes it possible to assess the severity of the disease in a specific population, and its burden and potential cost to the society.

Reproducibility of liver stiffness measurements

Apart from the ease of use, transient elastography has been shown to be highly reproducible in a series of 200 patients with 800 examinations performed (Fraquelli et al., 2007). The overall inter-observer and intra-observer agreement intraclass correlation coefficient (ICC) was 0.98 for both raters. However, there were several factors associated with significantly decreased reproducibility rates, including the presence of steatosis, patients with increased BMI, and in patients with lower degrees of liver fibrosis.

Limitations

Although transient elastography is an easy and rapid procedure, strict adherence to quality criteria should be followed to ensure the reliability of the results obtained. The IQR of all the readings should not exceed 30% of the final result (the median value), and the success rate of the scans should be at least 50%. The results should always be interpreted by a qualified clinician according to the clinical context, taking into account the patient demographics, disease etiology, and laboratory parameters. If the liver stiffness value appears to be discordant with the clinical scenario, then it is recommended to repeat a scan or proceed to a liver biopsy.

There is an estimated 5% patient failure rate in which a valid measurement cannot be obtained. This may be due to either low success rate, inability to obtain 10 valid acquisitions, or a high IQR/median liver stiffness ratio. In a study of 2,114 examinations, failure was seen in 4.5% of cases. The only significant factor associated with failure after multivariate analysis was a body

BMI of over 28, whereas operator, gender, or transaminase levels were not factors that were associated with failure in this particular series (Foucher, Castera et al., 2006). In addition to a high BMI, other factors associated with failure include adipose tissue or a thoracic fat belt overlying the right lobe of the liver. Presumably this prevents both the transmission of the vibration impulse through the thickened subcutaneous tissue and detection of the shear wave propagation through the liver. On the other hand, failure may also occur in very thin patients where the intercostal spaces are narrow, preventing proper probe placement in between the ribs. This is observed more commonly in thin Asian female subjects.

In another recent large series of 13,369 examinations over a 5 year period, the failure rate (zero valid acquisition) and rate of unreliable results (defined as fewer than 10 valid shots, and IQR/median liver stiffness of greater than 30%, or a success rate of less than 60%) was 3.1% and 15.8% respectively (Castera et al., 2010). The main reasons of failure to obtain a valid result and unreliable results include obesity (especially with increased waist circumference), and limited operator experience. Therefore, adequate training is important so that the failure rates are kept to a minimal, although there will still be a small proportion of patients who will not be suitable for liver stiffness measurement because of their body habitus.

Other factors may affect the liver stiffness value, reducing the diagnostic accuracy. As we have shown, both severe flares of hepatitis (ALT>10x ULN) and mild-to-moderate elevations of ALT may increase liver stiffness results. Whether steatosis increases liver stiffness is debatable. In studies of chronic hepatitis C, steatosis did not appear to affect liver stiffness values (Sandrin et

al., 2003; Ziol et al., 2005). Even in a study of non-alcoholic fatty liver disease, liver stiffness correlated with fibrosis but not steatosis (Yoneda et al., 2008). However, in healthy subjects, the presence of metabolic syndrome is associated with slightly higher levels of liver stiffness (Roulot et al., 2008). Other studies have shown steatosis to affect liver stiffness. In non-diabetic patients with genotype 1 chronic hepatitis C, insulin resistance contributed to liver stiffness independent of liver fibrosis (Petta et al., 2009). In CHB, steatosis may also increase liver stiffness (Y. G. Zhang, Wang, Wang, & Ou, 2010). Therefore, the true extent in how steatosis may affect liver stiffness remains to be fully determined. Recent studies have also shown that extrahepatic cholestasis may also increase liver stiffness (Millonig et al., 2008). Despite the larger sampling area of transient elastography compared to liver biopsy, the heterogenous nature of liver fibrosis in some disease entity (eg. cystic fibrosis, sclerosing cirrhosis, drug-induced hepatotoxicity) may potentially affect the accuracy of liver stiffness measurement.

Finally, the validation studies have used liver biopsies as the benchmark comparison for non-invasive tests including transient elastography. However, as described in the introductory chapters, liver biopsy remains an imperfect gold standard (and hence an imperfect reference standard), with a diagnostic accuracy which is closer to 80% for the majority of biopsy specimens. Therefore, the discordant results between liver biopsies and liver stiffness measurements may be due to discordance attributed to either liver biopsy or transient elastography, and lead to underestimation of the accuracy of these non-invasive tests.

CONCLUDING REMARKS

Over the past 5 years, significant progress has been made in the use of transient elastography in clinical practice. Despite the absence of consensus guidelines regarding the use of liver stiffness measurements in clinical practice, transient elastography is already widely used in many places, including Hong Kong. This widespread use is probably the consequence of patients and clinicians not wanting or advocating liver biopsies respectively. We have been able to determine the prevalence of significant fibrosis in a CHB population using this non-invasive technology, heightening the awareness of this disease to both patients and clinicians. Transient elastography has been shown to have an excellent negative predictive value but poor positive predictive value in diagnosing cirrhosis. We have been able to show that not only does severe elevation of ALT affect liver stiffness, but even mild elevation of ALT can also affect the liver stiffness values. In fact, liver stiffness has been shown in our study to correlate well with not only ALT, but also with several other routine liver enzymes. Therefore, elevation of ALT can have a confounding effect on liver stiffness values in estimating the degree of fibrosis. In addition to the assessment of liver fibrosis, our center has demonstrated that liver stiffness measurement may have a longitudinal role in assessing disease progression, therapeutic response, and in predicting liver-related complications. The main focus now should be on the development of validated guidelines on the use of transient elastography, and to incorporate this technology into current management guidelines of CHB.

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