NOVEL MARKERS OF LIVER FIBROSIS

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ABSTRACT

With chronic liver disease rising, the need to stage of liver disease and fibrosis accurately is paramount as it helps guide therapy and informs prognosis. Liver biopsy is a flawed gold standard, associated with morbidity and mortality. Application of simple non-invasive tests to assess fibrosis could provide a safe way of identifying patients in greatest need of intervention and of monitoring response to therapy.

I have shown in this thesis that transient elastography is an excellent tool for ruling out significant fibrosis in patients with chronic liver disease. It is easy to learn and successful scanning correlates well with histological liver fibrosis.

I have also shown that Use of APRI with a cut off of >1.5-2 and Fib-4 >3.25 can provide prognostic value for overall and liver-related mortality in patients with viral hepatitis.

Finally I have assessed a range of potential new biomarkers showing that combining measuring serum levels of the chemokine CXCL10 and the endothelial adhesion receptor VAP-1 can increase the correlation strength with fibrosis stage. Using morphometric analysis of liver fibrosis I show that the same markers can be linked to quantitatively measured fibrosis, removing subjective bias and reducing inter and intra-operator variance in histological assessment.

DEDICATION

My thesis is dedicated to my wonderful wife Emma, my two fantastic boys Thomas and Rory as well as my parents Elisabeth and Richard and my sister Anna. Without their love and support, I would not be where I am now

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ABBREVIATIONS

ALD Alcoholic liver disease
ALT – alanine aminotransferase,
APRI AST to platelet ratio index
AST – aspartate transaminase,
AST, aspartate transaminase;
AUROC, area under receiving operating characteristic;
av – average,
BMI, Body mass index;
CAP controlled attenuation parameter
CCL CC Chemokine ligand
CCL4 carbon tetrachloride
CCR chemokine (C-C motif) receptor
CD cluster of differentiation
CI Confidence Interval
CK-18 Cytokeratin-18
CPA Collagen proportional area
CT Computed tomography
CXCL C-X-C motif chemokine
CYP450 cytochrome P450 2E1
DMSO dimethyl sulphoxide
ECM extracellular matrix
ELF- Enhanced liver fibrosis test,
ELISA Enzyme-linked immunosorbent assay
EMT Epithelial mesenchymal transition
FASL Fas ligand
FFA free fatty acids
FGFB Fibroblast growth factor basic
FLC free light chains
FSP-1 fibroblast-specific protein 1
GCLP good clinical laboratory practice
GCSF granulocyte colony-stimulating factor
GMCSF granulocyte macrophage colony-stimulating factor
GP130 Gylcoprotein 130
HBV – Hepatitis B,
HCC, hepatocellular carcinoma;
HCV – Hepatitis C virus,
HGF Hepatocyte Growth Factor
HIV- Human Immunodeficiency virus,
HOMA-IR Homeostatic Model Assessment of Insulin Resistance
HR Hazard Ratio
HSC Hepatic stellate cells
I-TAC Interferon-inducible T cell alpha chemoattractant
IFN- γ Interferon gamma
Il Interleukin

IP10 Serum Interferon-gamma-inducible protein 10
IQR, interquartile range;
IR Insulin Resistance
ITU – Intensive care,
JNK c-Jun N-terminal kinase
LKK I kappa B kinase
LSM – Liver stiffness measurement,
LSM, liver stiffness measurement;
MCP-1 monocyte chemotactic protein -1
MMP matrix metalloproteinase
MRE magnetic resonance elastography
MRI Magnetic Resonance Imaging
MRS Magnetic resonance spectrography
NAFLD – Non-alcoholic fatty liver disease,
NASH Non-alcoholic steatohepatitis
NK Natural killer
NOS nitric oxide species
NPV, negative predictive value;
OR Odds ratio
P-Prospective,
P3Np N-terminal procollagen 3 peptide
PAI-1 plasminogen activator inhibitor 1
PBC, primary biliary cirrhosis,
PDGF platelet derived growth factor
PPV, positive predictive value;
PSC – primary sclerosing cholangitis,
R-Retrospective
RANTES Regulated upon Activation, Normal T cell Expressed and Secreted
RFA radio-frequency ablation
ROC, receiver operating characteristic;
ROS reactive oxygen species
RR relative risk
SD Standard deviation
SE Standard Error
SMA smooth muscle actin
TE, transient elastography
TG Triglyceride
TGF- β transforming growth factor –beta
TGF-α transforming growth factor alpha
TIMP tissue inhibitor of metalloproteinase
TNF- α tumour necrosis factor – alpha
TPA tissue plasminogen activator
TRAIL TNF – related apoptosis inducing ligand
VAP-1 Vascular Adhesion Protein 1
VEGF Vascular endothelial growth factor

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

Worldwide, liver related morbidity and mortality continue to rise. It is the 5th highest cause of death in the UK (Office National Statistics)

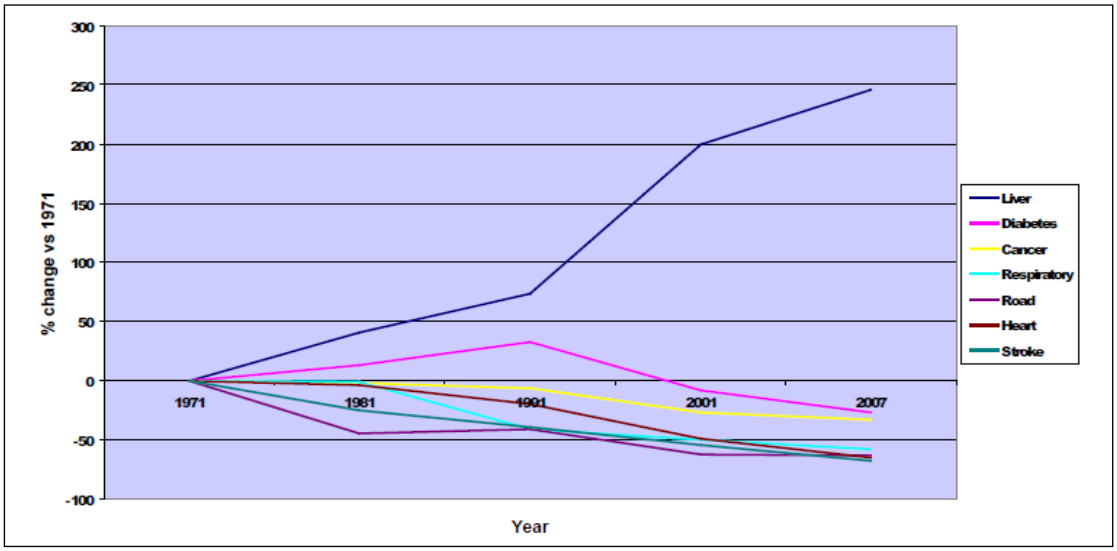


Figure 1-1 Increasing Liver Mortality – data from ONS

Cirrhosis is a pathological process common to all causes of chronic liver disease. The exact point when fibrosis becomes irreversible is difficult to define and the underlying mechanisms are not completely understood. Cirrhosis has major effects on the liver causing a disordered architecture leading to portal hypertension. This manifests itself as varices, ascites and encephalopathy. Cirrhosis also has the potential to develop into hepatocellular carcinoma (HCC) in 3% per year in HBV and HCV with similar risk in alcoholic liver disease. In patients with iron overload and cirrhosis, this can rise as high as 7% per year. Risk drops with venesection but not back to baseline. In contrast, patients with autoimmune hepatitis related cirrhosis have a very low risk of HCC development(Ryder, 2003). Patients with

established cirrhosis can remain free of clinical symptoms for many years, in which case they are said to have compensated cirrhosis. The rate at which both fibrosis and subsequent decompensation develop are determined by environmental and genetic factors which helps to explain the broad spectrum of response to the same aetiological agent in patients with chronic liver diseases(Bataller and Brenner, 2005). Patients with self-limiting acute disease such as hepatitis A do not usually develop scarring despite activation of inflammatory pathways and scarring only develops if the injury becomes chronic and persists.

The development of clinical complications of portal hypertension or symptoms and the development of HCC are associated with a much worse outcome as illustrated below(D'Amico et al., 2006).

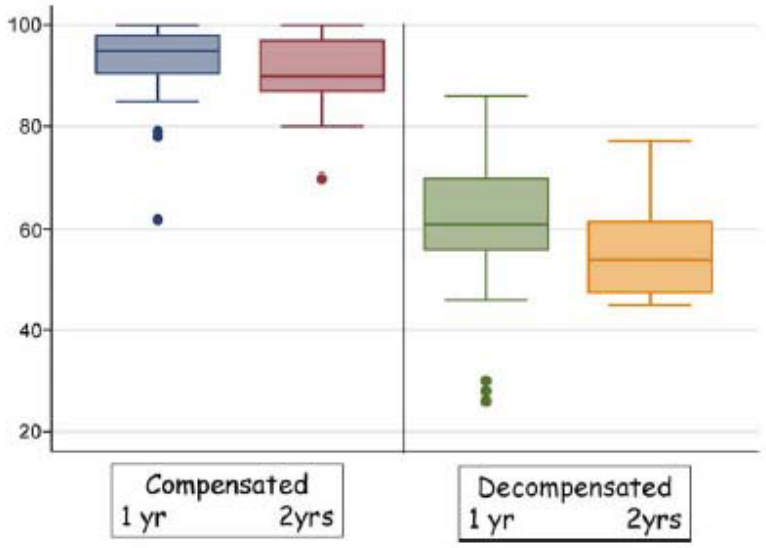


Figure 1-2 Chart showing worsening prognosis after decompensation (from D'Amico et al, J Hep 2006)

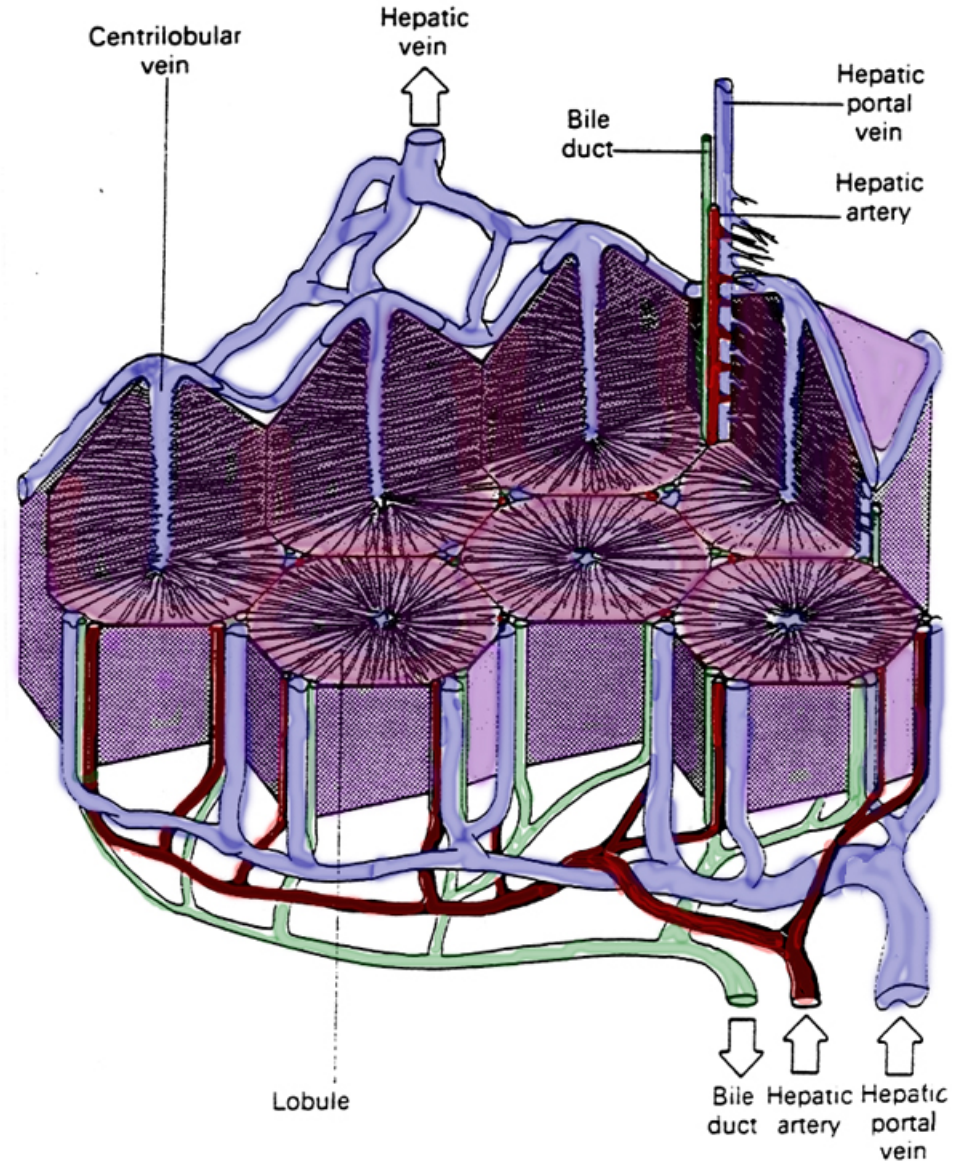
Liver transplantation is currently the only curative treatment for end stage chronic liver disease. Its high demand has not been matched by a rise in liver donations leading to high levels of mortality on the waiting list and those who receive a liver transplant require life-

long immunosuppression with the consequent risks of infection cancer and increased cardiovascular death and renal impairment.

1.2 Normal liver architecture

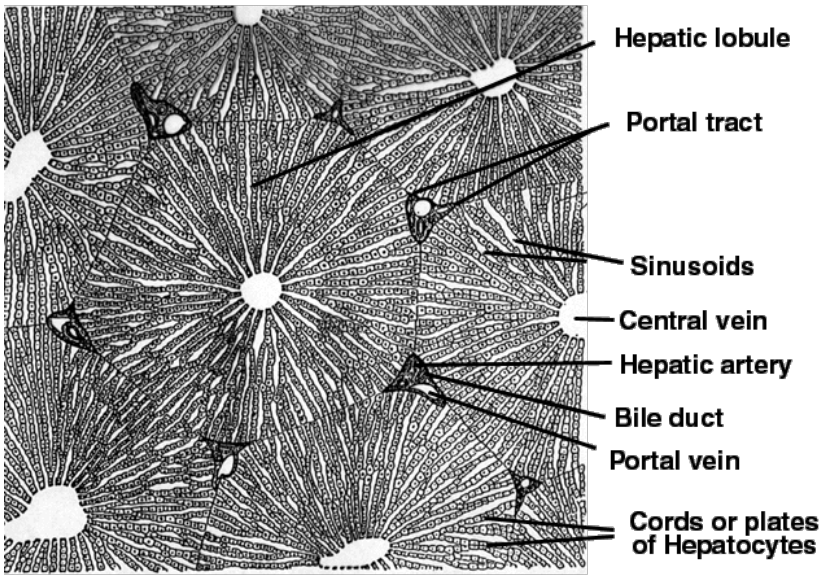
A fibrocollagenous capsule surrounds the liver with connective tissue (stroma) extending into it as septa, which contain the vasculature and ducts of the liver. The stroma thus divides the liver into lobules and allows for an ordered architecture supporting veins, arteries and a biliary system. This architecture becomes disrupted in fibrotic liver disease with consequent effects on the flow of blood and bile in and through the liver. The replacement of normal liver cells with scar tissue also has functional consequences that can result in liver failure if the mass of functional liver cells falls below a threshold.

The diagrams below help to illustrate this normal architecture.



Taken from https://courses.stu.qmul.ac.uk/smd/kb/microanatomy/d/alimentary/answers/index.htm

Figure 1-3 Diagram to illustrate normal flow of blood and bile through the liver



Taken from https://courses.stu.qmul.ac.uk/smd/kb/microanatomy/d/alimentary/answers/index.htm

Figure 1-4 Alternative view of normal liver architecture showing portal tract, sinusoidal and central vein relationships

1.3 What is the process behind liver fibrosis?

Repetitive injury in the liver stimulates an inflammatory response, which attempts to clear the instigating factor, for example a virally infected hepatocyte, and then heal the injury through fibrogenesis to maintain hepatic integrity. Liver fibrogenesis occurs in the context of a chronic inflammatory response, which leads to myofibroblast proliferation, increased production of extracellular matrix and parenchymal cell proliferation as part of a process of scar formation and regeneration that replaces hepatocytes lost through necrosis or apoptosis. In the wound healing response, increased extracellular matrix (ECM) deposition occurs and this is a normal appropriate response. The constituents of this matrix include basement membrane and interstitial collagens, proteoglycans, elastin and matrix glycoproteins such as fibronectin and laminin. In normal liver fibril forming collagens are physiological. Types 1, 3 and 5 are found in the capsule, around large vessels and in the portal areas(Friedman, 2008)

Types 3 and 4 collagens with fibronectin accumulate early in liver injury in the space of Disse and over time greater amounts of type 1 and 4 collagen together with elastin and laminin re deposited as the injury becomes sustained and chronic. Although all 4 types of collagen are increased by 3-10 fold, the proportion of type 1 collagen in chronic liver disease increases the most. The maturity of the fibrosis can therefore be assessed using knowledge of the different types of ECM deposited together with the amount of crosslinking observed. Recently it was shown that elastin accumulation in liver injury occurs not only as a result of increased synthesis but also as a failure of matrix metalloproteinase (MMP) -12 derived degradation(Pellicoro et al., 2012).

1.4 Development of cirrhosis

Wound healing is a complicated process, which involves matric deposition, degradation and remodelling. Excessive accumulation of the ECM occurs in persisting hepatic injury associated with dysregulated regeneration. This leads to disruption of normal tissue architecture and loss of function. Subsequent development of nodules of regenerating hepatocytes surrounded by scar tissue defines cirrhosis. An imbalance between fibrinogenesis and fibrinolysis leads to scar formation. Fibrogenesis is the production of ECM whereas fibrinolysis is the breakdown of ECM by matrix metalloproteinases. Fibrinogenesis is usually insidious taking many years for cirrhosis to manifest other than in special situations such as neonatal liver disease.

There are many different causes of liver injury with different patterns of damage reflecting the site of injury (e.g. biliary, perisiunsoidal or peri central). However the underlying processes are similar and chronic inflammation is central to the process of fibrogenesis regardless of the aetiology of liver disease. The different patterns of liver injury are described below.

1.5 Crosslinking and development of advance cirrhosis

Crosslinking of collagen and maturation of the scar with increasing levels of elastin occurs via the action of lysyl oxidase and tissue transglutaminase. In animal models the reversibility of fibrosis is dependent upon this level of cross-linking and increased septal thickness / smaller nodules are associated with poor outcomes (Nagula et al., 2006).

1.5.1 Why is cirrhosis important?

As mentioned above, the disruption to the liver in cirrhosis presents structural and physiological problems to the patient. Patients with cirrhosis often have portal hypertension as a result of intrahepatic resistance and increased portal blood flow. The resistance to blood flow is due to the fact that the normally compliant liver cannot respond to increased portal blood flow by distending because the scar and regenerative nodules compress the vasculature. This manifests as varices, ascites and splenomegaly.

Replacement of hepatocytes with scar tissue reduces the functional mass of liver tissue and leads to liver failure with reduced formation of essential proteins involved in clotting as well as reducing the liver's ability to filter splanchnic blood. In patients with cirrhosis and varices, one-third will bleed over a two-year period(NIEC, 1988)

1.6 Cell death

Apoptosis maintains tissue homeostasis and health by counterbalancing cell proliferation and eliminating damaged and or old cells. Liver injury frequently results in hepatic cell apoptosis. Any imbalance of this process will lead to liver pathology.

Apoptosis can be triggered by several intra and extra cellular triggers. Intracellular triggers include DNA damage that results in mitochondrial permeability and the release of pro-apoptotic factors. Extracellular signals are mediated through cell surface receptors particularly those belonging to the tumour necrosis factor receptor superfamily hat can be activated by their ligands including TNF- α , TNF related apoptosis inducing ligand (TRAIL) CD40L and Fas ligand (FasL)(Malhi et al., 2010). In particular, activation of Fas and TNF-R1 is associated with hepatocyte apoptosis in a wide variety of liver diseases, including viral hepatitis, fulminant hepatic failure, cholestatic liver disease, alcoholic hepatitis, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), Wilsons' disease and ischemia-reperfusion injury(Akazawa and Gores, 2007). Kupffer cells are located in the hepatic sinusoids and from a component of the reticuloendothelial system. They take up particulate and bacterial products from the portal blood and can secrete an array of cytokines that modulate local inflammatory responses. Clearance of apoptotic bodies by Kupffer cells triggers their release of profibrogenic cytokines such as transforming growth factor -beta (TGF- β) and TNF- α. Hepatic stellate cells which are located beneath the sinusoidal endothelium within hepatic sinusoids are also able to engulf apoptotic bodies derived from hepatocytes leading to their production of TGF-β and type 1 collagen(Canbay et al., 2003) and NADPH that consequently generates free radicals.

Other forms of cell death include necrosis and autophagy. Extensive hepatocyte necrosis is a feature of ischaemic injury, associated with excessive reactive oxygen species (ROS) production, paracetamol poisoning and fulminant liver failure. The cytochrome P450 2E1 (CYP2E1) has an important role in the generation of ROS(Nieto et al., 2003). Hepatic stellate cells (HSCs) cultured in the presence of CYP2E1-overexpressing (E47) HepG2 cells produce more collagen. This may help to explain in part the pathogenesis of alcoholic liver disease as CYP2E1 is induced by alcohol.

Autophagy is a catabolic process dependent on lysosomes for execution and occurs under conditions of nutrient depravation and is linked to cell survival.

1.7 What cells are involved and how is the process regulated?

Several lineages of fibroblasts are implicated in fibrogenesis including stellate cells portal fibroblasts and bone marrow derived fibroblasts. Differentiated myofibroblasts are the main source of excessive ECM production, but their origin is unresolved and several distinct myofibroblast precursors have been described and are summarised in the figure below(Brenner et al., 2010).

1.7.1 Resident Cells

The most accepted myofibroblast progenitors are HSCs located in the space of Disse below the sinusoidal endothelium where they act as pericytes. HSCs are also known as lipocytes, Ito cells, or peri-sinusoidal cells. They represent a major reservoir of vitamin A in the human body. In response to cytokines and growth factors released during injury quiescent stellate cells transform into an activated state during which they secrete extra cellular matrix and express smooth muscle actin (α-SMA), which although not tissue specific can be used to identify activated myofibroblasts with contractile properties. The contractile properties of HSCs regulate portal resistance and blood flow as mentioned earlier. Activated stellate cells produce cytokines, chemokines such as monocyte chemotactic protein -1 (MCP-1) and growth factors including and increase their expression of platelet derived growth factor (PDGF) receptor and TGF-β receptors. Loss of intracellular retinoid is a notable feature of stellate cell activation, but it is uncertain if this is required for their activation.

Other sources of liver myofibroblasts include portal fibroblasts and second layer fibroblast located around the centrolobular vein.

1.7.2 Epithelial mesenchymal transition (EMT)

This is a second potential (and controversial) source of fibroblasts or myofibroblasts. EMT proposes that hepatocytes and cholangiocytes can differentiate into fibroblast like cells that lay down matrix. EMT occurs when epithelial cells lose key epithelial characteristics becoming more motile and in some cases fibrogenic. Demonstrating this change is difficult however because of a lack of definitive markers and doubts over the specificity of previous markers such as fibroblast-specific protein 1 (FSP1 also known as S100A4) which has been used to define EMT in vivo(Osterreicher et al., 2011). Recently Taura et al suggested that hepatocyte EMT seen in vitro is a function of the combination of TGF-b treatment and culture conditions which is not seen in vivo(Taura et al., 2010). However, before discounting EMT it should be noted that the murine carbon tetrachloride (CCl4) model used does not accurately model human disease.

The question of cholangiocyte EMT is also open to question. Several groups looking at human and animal models have reported that cholangiocytes in fibrotic livers coexpress multiple epithelial and mesenchymal markers, suggesting that they were likely to be undergoing EMT(Wells, 2010). However lineage tracing of cells in vivo suggests that very little EMT occurs in either hepatocytes or cholangiocytes. After inducing liver fibrosis by varying means, no resultant myofibroblast originated from genetically marked epithelial cells(Chu et al., 2011, Kisseleva and Brenner, 2011).

1.7.3 BM derived cells

Fibrocytes are a population of CD45+ leukocytes derived from the bone marrow and are inactive mesenchymal cells. In culture they can differentiate into myofibroblasts and have been implicated in fibrogenesis in the skin, kidneys, lungs and liver. Upon injury they

migrate to the site of injury and once there they secrete TGF-B and MCP-1 that promote ECM deposition. The contribution of fibrocytes to the myofibroblast population is minor at probably only 3-5%(Brenner et al., 2010).

1.8 Important cytokines in liver fibrosis

These molecules are important mediators of the activation of stellate cells and can be split into two classes(Tsukamoto, 1999, Friedman, 2000)

Broadly speaking the relevant mitogenic (stimulate cell proliferation) cytokines are:

PDGF

CC Chemokine ligand 2 (CCL2) (or MCP-1)- promigratory

TGF-A

Interleukin-1 (Il-1)

TNF- α

The main fibrogenic (induces matrix proteins) cytokine is TGF-B

1.8.1 Mitogenic

PDGF

PDGF is the most potent mitogen for HSCs and other cells of mesenchymal origin. It is up regulated in the fibrotic liver. There are 4 subtypes (A-D) and two structurally related tyrosine kinase receptors: PGDFR-α and PDGFR-β. Injury is associated with increased autocrine PGDF and up regulated PDGF-R. Inhibition of PDGF attenuates liver fibrinogenesis experimentally by reducing alpha-SMA and Type 1 collagen expression(Borkham-Kamphorst et al., 2004).

CCL2 (MCP-1) is a chemokine that is up regulated during hepatic inflammation and which recruits monocytes and macrophages to sites of inflammation through its activation of chemokine receptor (CCR) 2. Elevated levels correlate with the number of leukocytes infiltrating portal tracts in one study(Marra F and M, 1998)

TGF-α and Il-1

TGF-α is produced by macrophages and induces epithelial development. It is closely related to epidermal growth factor (EGF). It acts synergistically with IL-1(Dinarello, 2000) which is rapidly expressed in response to tissue injury. Il-1 is an important participant with other cytokines in controlling the progression of liver injury to fibrosis.

TNF-α

TNF-α activates several intracellular pathways to regulate cell death, proliferation and inflammation. It also contributes to hepatocyte proliferation and liver regeneration. It is secreted by macrophages and to a lesser extent fibroblasts and acts via two receptors TNF-R1 and TNF-R2. The dichotomous nature of action is dependent on one pathway inactivating the other. The process of regulation involves the I kappa B kinase (IKK), ROS and c-Jun N-terminal kinase (JNK) pathways and is summarised in a review by Schwabe et al.(Schwabe and Brenner, 2006)

1.8.2 Fibrogenic

TGFB1 is a highly profibrogenic cytokine with mitogenic properties. It is produced by multiple liver cell types including stellate cells, Kupffer cells, hepatocytes and cholangiocytes. TGFB1 is critical for the proliferation and activation of fibrogenic myofibroblasts. There are many sources, but autocrine expression is the most important. Perhaps surprisingly, as the degree of liver fibrosis increases, levels of TGFB1 are observed to decrease with a corresponding elevation in HGF(Imbert-Bismut et al., 2001) and over expression of HGF in cell lines supresses increases of TGFB1(Ueki et al., 1999).

The therapeutic inhibition of TGF-B is complicated by the potential for promoting inflammation and cancer or reducing apoptosis.

1.8.3 Anti-fibrotic cytokines

Interferon gamma (IFN- γ)

This cytokine is widely know for its anti-viral properties and can directly inhibit viral replication. It is produced by natural killer (NK) cells and subsets of T cells

Interferon gamma has been shown to decrease collagen synthesis as well as inhibiting the activation of HSCs(Baroni et al., 1996). It also reduces the expression of type 1 and 4 collagen and fibronectin in HSCs in vitro. An RCT of therapy in HCV patients showed no benefit (Pockros et al., 2007).

Il-10

This anti-inflammatory cytokine is released by monocytes, some populations of dendritic cells, macrophages and subsets of T cells. It down regulates production of inflammatory cytokines such as Il-1 as well as profibrogenic cytokines such as TGF-B. Il-10 deficient mice show increased susceptibility to hepatic fibrosis due to CCL4 and helminthic infection (Louis et al., 1998, Mentink-Kane et al., 2011). In humans with hepatitis C virus (HCV) infection, administration of IL-10 was shown in a pilot study to reduce hepatic inflammation and fibrosis(Nelson et al., 2000). Several years later, however this study showed that although fibrosis improved, viral loads increased due to alterations in immunologic viral suppression(Nelson et al., 2003).

Hepatocyte Growth Factor (HGF)

HGF in animal models has been shown to have antifibrotic effects through suppression of TGF-beta mediated transcription of type 1 collagen and activated HSCs(Ozaki et al., 2002). Studies in humans are complicated by HGF's potent mitogenic activity

1.9 Diseases associated with liver fibrosis

The pattern of damage to the liver and the symptoms experienced depend on the aetiology of the disease and also the type of cell targeted by the liver injury.

Fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of disease ranging from the most benign hepatic steatosis through to steatohepatitis with in some cases progression to fibrosis and cirrhosis. It is a manifestation of the metabolic syndrome associated with diabetes, dyslipidaemia and obesity and the prevalence is rising along side the rise in obesity and diabetes. It now represents a major cause of liver disease worldwide and perhaps the most common type of liver disease in western countries(de Alwis and Day, 2008). The diagnosis cannot be made on histology alone because the histological features are indistinguishable from alcoholic liver disease.

Non-alcoholic steatohepatitis (NASH) is characterised by a spectrum of inflammation, apoptosis/ necrosis and fibrosis. It is highly likely that insulin resistance is instrumental in the pathogenesis of NASH, but how this triggers steatohepatitis is unknown. Progression to steatohepatitis and fibrosis depends on inflammatory pathways activated by free fatty acids (FFA), inflammatory cytokines and adipokines, oxidative stress and mitochondrial dysfunction in a complex interplay with genetic predisposition (Dowman et al., 2010).

Histologically, steatohepatitis is characterized by steatosis with mixed lobular inflammation and hepatocyte ballooning (±Mallory's hyaline) in the presence or absence of fibrosis. Ballooning is a morphological feature of apoptosis.

Alcoholic liver disease (ALD)

Steatosis and steatohepatitis seen in alcoholic liver disease are histologically indistinguishable form the changes seen in NAFLD and thus the diagnosis rest on evidence of alcohol excess.

Alcohol induces an increase in lipid peroxidation by enhancing the production of ROS and decreasing the level of endogenous antioxidants(Ishii et al., 1997).

The break down of alcohol within hepatocytes produces ROS and acetaldehyde, both of which have pro-fibrotic properties on HSCs via a paracrine mechanism(Purohit and Brenner, 2006).

The inflammatory response to alcohol is not however universal and likely to be multifactorial as with NAFLD, i.e. with environmental and genetic predisposition.

Interestingly alcohol actually has a damping effect on NK cells (which themselves kill activated stellate cells). This could account for the accelerated nature of fibrosis seen upon cessation of alcohol intake.(Jeong et al., 2008)

Cholestatic liver disease

Extrahepatic cholestasis is due to bile duct obstruction. Classically this is associated with oedema, proliferation of bile ductular structures and a neutrophilic ±lymphocytic / plasma cell infiltrate. This reaction is known as a ductular reaction and is common in biliary obstruction.

Intrahepatic cholestasis may be drug or toxin related, genetic due to functional bile flow impairment (e.g. mutation of bile salt transport proteins) and due to immune mediated destruction of small intrahepatic ducts by primary sclerosing cholangitis (PSC), primary

biliary cirrhosis (PBC) PBC and other vanishing bile duct syndromes. In PBC the damage is immune related and associated with a T cell infiltrate of intrahepatic bile ducts. This process leads to bile duct destruction and ductopenia. The process if left unchecked leads to portal tract expansion by fibrosis with ultimately portal bridging(Jones, 2007).

Chronic Viral Infection

The pathogenesis of chronic viral induced fibrosis is poorly understood due to the lack of animal models of chronic viral hepatitis infection. HCV eludes the host immune system and infects hepatocytes. This leads to a slow process of hepatocyte injury with oxidative stress and apoptosis leading to HSC activation and ECM deposition. Typically parenchyma inflammation is seen on histological examination although this may be modest consistent with the slow progression of injury and fibrosis.

1.10 Matrix Degradation

Stellate cells express metalloproteinases required for matrix degradation including MMP-2 and MMP3. Markedly increased expression of MMP-2 is characteristic of cirrhosis(Benyon et al., 1996). Degradation of ECM hastens its replacement by fibril-forming collagen, which further activates stellate cell growth and MMP-2 production. The collagenases are inhibited by tissue inhibitor of metalloproteinase 1 and 2 (TIMP-1 and 2). The increased expression of TIMPs shifts the balance of extracellular matrix towards deposition of collagen and fibrosis. This up regulation requires activated stellate cells as the relevant receptors are not seen on quiescent HSCs. Sustained TIMP-1 expression is a key reason for progressive fibrosis and its reduction is therefore a key in resolving fibrosis and allowing scar resolution.

1.11 Reversion to quiescence?

Removing the underlying cause of liver injury is the most effective way to prevent fibrosis such as in haemochromatosis or Wilsons disease or alcoholic liver disease. Cirrhosis had traditionally been considered an irreversible state but recent evidence has shown that the development of fibrosis is dynamic and potentially reversible depending on the extent of cross-linked elastin and collagen. Resolution of liver fibrosis is associated with reversion of activated HSCs to quiescence or perhaps by altering the balance of proliferation to cell death (apoptosis). Stellate cells apoptosis has been observed in rats recovering from CCL4 injury(Iredale et al., 1998). Degradation of matrix appears also to promote HSC apoptosis. In vitro cells treated with MMP-9 stimulate HSC apoptosis(Zhou et al., 2004)Treatment of these cells with TIMP-1 exerts and anti-apoptotic effect by inhibiting MMP activity(Murphy et al., 2002). The activated stellate cell by secretion of collagen and TIMP protects itself against apoptosis, but the default pathway for HSC may be for them to undergo apoptosis(Issa et al., 2001). With resolution of fibrosis, the HSC survival signals are removed and they undergo apoptosis, further reducing TIMP, increasing MMP and leading to histological resolution(Elsharkawy et al., 2005).

1.12 Liver biopsy and its flaws

Liver biopsy allows a physician to make a diagnosis and assess the severity of the liver disease including inflammation, necrosis, steatosis or deposits of iron and copper as well as fibrosis and architectural changes. Liver biopsy is however not without its limitations. Several scoring systems are used to assess fibrosis but they are all flawed because the score represents numerical shorthand for a categorical assignment. It thus neither represents integers nor numerical measurements along a continuum in a mathematical sense (Standish et al., 2006). The scoring system is a pathologist's way of describing more than just fibrosis. It contains a mixture of features and at higher stages can represent more architectural change and nodularity rather than the amount of fibrous tissue; hence the degree of collagen seen in similar staged biopsies may vary. It is thus incorrect to assume that the scoring system is describing a continuous variable (fibrosis) using a categorical system and that the stages assume linearity in progression of fibrosis, which does not reflect the disease progression.

Liver biopsy is associated with pain (20% of cases) (Cadranel et al., 2000) and rarely major side effects such as bleeding and infection (in 3%(Piccinino et al., 1986). Furthermore, it only samples a small portion of the liver (1/50,000) and interpretation of histology is open to intra and inter-observer error(Bedossa et al., 2003). Pathologists have to assess the >1kg liver based on a piece of tissues weighing 10-15mg hence patchy disease may be over or under reported due to variations between lobules(Rosenberg et al., 2004). One study of HCV patients assessed biopsies taken from the left and right liver lobes laparoscopically. 33% of patients had at least one stage difference between the lobes and that sampling error could lead to under diagnosing of cirrhosis in 14.5% of the patients. This would have major

implications for the patient in terms of management and follow up(Regev et al., 2002). This is perhaps where serum based scoring systems may in future be more widely utilised.

1.12.1 What makes an "adequate" liver biopsy?

Until recently, a liver biopsy containing six portal tracts satisfied histopathologists(Bravo et al., 2001). Recently the American Association for the Study of Liver Disease released guidelines on what constitutes an adequate biopsy. This was >20mm in length and >11 portal tracts(Rockey et al., 2009). To achieve this it is likely that 3 or 4 passes are needed. Recent UK data has suggested that only 4% of samples measured 20mm or more and of those, 2% had 11 or more complete portal tracts(Naseer et al., 2011). It was also noted in the recent paper by Boursier as part of a multicentre study looking at 1785 patients that liver biopsy length had no influence on the diagnostic accuracy of non-invasive markers(Boursier et al., 2012a).

1.12.2 Repetitive liver biopsy

Despite the good correlation between fibrosis staging and specific outcomes (time to develop cirrhosis, time to develop clinical decompensation), the progression of liver fibrosis over time is commonly not linear(Hintermann et al., 2010) hence the need for repeated measurements to give an idea of disease behaviour. It is neither practical nor ethical to undertake frequently repeated liver biopsies for monitoring of disease progression or regression due to the reasons mentioned above.

1.13 Collagen Proportionate analysis to quantify fibrosis

Collagen proportional area (CPA) is a quantitative digital image analysis and was shown in 2009 to correlate with Ishak staging ($r=0.47$; $p<0.001$). For significant fibrosis (Ishak >2), the AUROC for CPA was 0.84 with a cut off of 6% giving 78% sensitivity and 80% specificity. For severe fibrosis (Ishak 5+6), the AUROC was 0.9 with a CPA value of 9% giving 78% sensitivity and 88% specificity(Calvaruso et al., 2009). The absolute values of collagen seen are summarised in an excellent review by Standish in the figure below. What is noted is that the increasing stage of fibrosis compared to Ishak stage of disease is not linear (Standish et al., 2006). The paper also illustrates that within each histological stage there may be great differences in CPA. Two biopsies may be Ishak stage 6 (cirrhotic) due to parenchymal nodules surrounded by fibrous tissue, but the proportion of collagen might differ vastly. The paper shows two cirrhotic biopsies with CPAs of12% and 27% respectively.

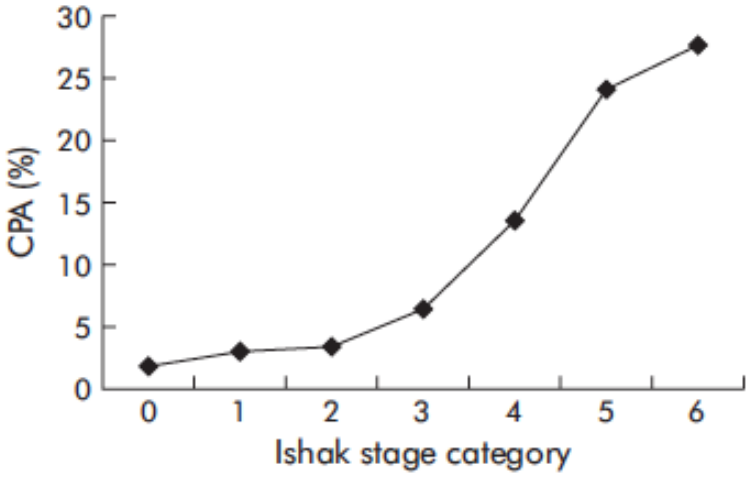


Figure 1-5 CPA vs. Ishak stage from Standish et al showing rise in CPA with fibrosis stage

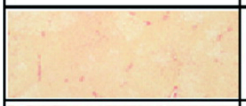
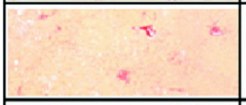
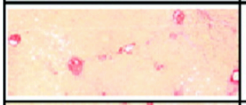
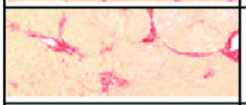
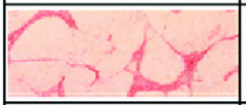
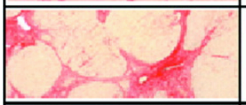
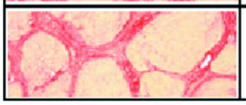
Appearance	Ishak stage: Categorical description	Ishak stage: Categorical assignment	Fibrosis measurement*
	No fibrosis (normal)	0	1.9%
	Fibrous expansion of some portal areas ± short fibrous septa	1	3.0%
	Fibrous expansion of most portal areas ± short fibrous septa	2	3.6%
	Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging	3	6.5%
	Fibrous expansion of portal areas with marked bridging (portal to portal (P-P) as well as portal to central (P-C))	4	13.7%
	Marked bridging (P-P and/or P-C), with occasional nodules (incomplete cirrhosis)	5	24.3%
	Cirrhosis, probable or definite	6	27.8%

Figure 1-6 Collagen proportional area vs. Ishak fibrosis stage

The accuracy of CPA of stained biopsy samples is dependent upon biopsy size. Recently, It was shown that when compared to a tissue block, biopsy samples 22-28mm in length give a 90% probability of CPA being within 5% of the block's CPA. For biopsies 12-15mm in length, this drops to 75%(Hall et al., 2013). Since the median biopsy length was 23mm it is likely that the CPA analysis represents with a high probability what degree of fibrosis is seen on the block.

1.14 Limits of Non-invasive markers

There is a resistance to the adoption of non-invasive markers for diagnosing fibrosis due to the lack of well-designed studies assessing the various methods. Where studies do exist there is a perceived lack of external validation especially as the number of tests is constantly expanding leaving little time to validate and test them in clinical practice. Another concern as mentioned above is the fact the gold standard (liver biopsy) is not ideal and prone to error, which makes benchmarking new markers difficult. Other drawbacks are that most current markers do not reflect liver fibrosis but necroinflammation and injury. There is also a paucity of data on using the models to monitor response to treatment or change in disease burden over time. Other weaknesses include intra-laboratory variations in serum marker assays with different reference ranges.

As discussed below a further weakness is that most of the studies (as mentioned above) compare groups of patients at the extremes of disease. Studies usually quote that the marker can differentiate minimal or no fibrosis from cirrhosis, but that assessing intermediate levels is less robust.

1.15 Assessing liver fibrosis

Any new method of assessing liver fibrosis must be judged against liver biopsy. Due to the misclassification rate of biopsy, it becomes impossible to achieve a perfect concordance with an area under receiver operating characteristic (AUROC) of 1. Assuming that liver biopsy has a sensitivity and specificity of >90% and the prevalence of fibrosis of ~40%, a perfect non-invasive test would only reach and AUROC of 0.9(Mehta et al., 2009) . Another consideration is that fibrosis in a liver biopsy is assessed by architectural changes rather than the amount of fibrosis therefore assuming a direct correlation between non-invasive value and fibrosis stage may be incorrect(Ratziu, 2010).

The next question pertaining to diagnosing fibrosis remains the ability of various markers to accurately predict the different stages of fibrosis. Most biomarkers currently available (see below) are accurate at identifying patients with significant fibrosis. This is critical, as patients with cirrhosis must be further assessed for complications of cirrhosis such as varices and hepatocellular carcinoma.

1.16 The ideal liver fibrosis biomarker

An ideal liver fibrosis marker should have the following characteristics: (Rossi et al., 2007)

- Liver specific
- Readily available and standardised between all laboratories performing diagnostic biochemistry / haematology
- Not subject to false positive results, for example due to inflammation
- Identifies the stage of fibrosis

1.16.1 Development of non-invasive liver fibrosis biomarkers

Serum markers for the prediction of fibrosis generally fall in to two categories.

The Indirect markers are a mixture of commonly observed biochemical abnormalities and clinical observations seen in liver disease, which evaluate synthetic dysfunction. They don't however assess fibrogenic cell changes. More recently, a greater understanding of the process behind liver fibrosis has prompted researchers to look at alternative markers(Martínez et al., 2011) Direct markers use the fact the fibrinogenesis and fibrinolysis generate peptides that can be measured in the peripheral circulation. It is noteworthy that TIMPs also appears in the circulation and their assay is part of some tests of fibrinogenesis. One caveat is that the peptides are also seen in tissue inflammation.

Identification of hepatic stellate cells was a key development in understanding the liver's response to injury. Activation of hepatic stellate cells is the primary event in hepatic

fibrogenesis. Quiescent cells transform into proliferative, fibrogenic, and contractile myofibroblasts. Fibrosis evolves when the balance between extracellular matrix degradation and production is disrupted(Bataller and Brenner, 2001). Of note, even though fibrosis is a local reaction of the liver to chronic injury, serum levels of fibrogenic cytokines, extracellular matrix proteins, and degradation products are markedly increased in cases of advanced fibrosis (bridging fibrosis or cirrhosis). Identification of these markers is therefore becoming of more interest(Martínez et al., 2011). It also helps to explain why fibrosis biomarkers lack sensitivity and specificity in the initial stages of liver fibrosis as they are affected by fibrosis in other organs and renal problems.

The most common markers used in current assays involve measuring products of extracellular matrix synthesis or degradation and the enzymes that regulate their production or modification: hyaluronic acid, serum collagenases, TIMP, and profibrogenic cytokines such as TGF- β1 (Rosenberg et al., 2004) or measure inflammatory markers such as cytokines and chemokines.

1.17 Current biomarkers

1.17.1 Indirect markers

Several biomarkers have been developed to look at fibrosis stage have been described using various combinations of tests listed below:

APRI (AST to platelet ratio index): Aspartate transaminase (AST) and platelets

FIB-4: alanine aminotransferase (ALT) and age

AST: ALT ratio

APRI

The APRI is calculated as (AST/upper limit of normal)/platelet count (10^9/l) x100. AST measures liver injury and the platelet count is an indicator of splenomegaly and portal hypertension. The sensitivity and specificity are dependant upon the cut off used and this varies from <0.5 to >2.0. It has been validated in HCV patients in a recent meta-analysis, but with only modest accuracy. For significant fibrosis, severe fibrosis and cirrhosis, the AUROC for APRI were 0.77, 0.8 and 0.83 respectively(Lin et al., 2011). This meta-analysis highlights the issue of papers quoting different cut off but suggests that an optimal cut-off for sensitivity and specificity is 0.7 with NPV of 79% and PPV of 70% for significant fibrosis.

Fib-4 was first described in 2006 by Sterling et al in an HCV cohort and is calculated as age [years] × AST [IU/L]/platelet count [expressed as platelets × 10^9/L] × ($ALT^{1/2}$[IU/L])(Sterling et al., 2006) The AUROC of the index was 0.765 for differentiation between Ishak stage 0-3 and 4-6. At a cutoff of <1.45 in the validation set, the negative predictive value to exclude advanced fibrosis (stage 4-6) was 90% with a sensitivity of 70%. A cut-off of >3.25 had a

positive predictive value of 65% and a specificity of 97%. Using these cutoffs, the authors state that 87%of the 198 patients with FIB-4 values outside 1.45-3.25 would have been correctly classified, and liver biopsy could be avoided in 71% of the validation group. Similar values were found in a French cohort a few years later(Vallet-Pichard et al., 2007).

AST: ALT

AST and ALT are enzymes both released into the circulation following damage to hepatocytes. The ratio between the two has been validated in several diseases, particularly ALD and NAFLD to correlate with severity of hepatitis and risk of fibrosis. A cut of >1 has been proposed as a test of cirrhosis with a 100% positive predictive value (PPV). The test had a 46.7 – 53.2% sensitivity and a negative predictive value (NPV) of 80.7 -88.1%(Sheth et al., 1998, Park et al., 2000)

Forns

The Forns score was developed in 2002 using an HCV cohort (Forns et al., 2002). Using a cut off of <4.2, the presence of significant fibrosis (F2-F4) could be excluded with an NPV of 96%. The AUROC was 0.86. It is calculated as: 7.811 - 3.131 × ln [number of platelets (10^9/l)] × 0.781 ln [GGTP (U/L)] + 3.467 × ln [age (years)] - 0.014 [cholesterol (mg/dl)]. This value is similar to that found a few years later in another HCV cohort of 228 patients. The AUROC for predicting ≥F2 fibrosis (METAVIR) was 0.913 (0.867-0.947)(Coco et al., 2007).

α2-macroglobulin

Is secreted by stellate cells and hepatocytes and is a major plasma protein. It is a broad inhibitor of endoproteases and elevated serum levels are seen in liver disease which correlate with the degree of liver fibrosis(Naveau et al., 1994).

Apolipoprotein A1

This protein is the major component of HDL and levels are negatively correlated with liver fibrosis(Poynard et al., 1991); it is also altered in poorly controlled diabetes, nephrotic syndrome and smoking.

Haptoglobin

This is an acute phase serum protein that binds free haemoglobin and is elevated in inflammatory conditions. Reduced levels are observed in later stages of fibrosis(Imbert-Bismut et al., 2001).

1.17.2 Direct markers

1.17.2.1 Glycoproteins and polysaccharides

Hyalauronic acid

This is a glycosaminoglycan present in extracellular matrix including the liver but also other tissues notably the joints; it is found in serum and synovial fluid. It is an essential component of the ECM and it is produced in the liver by stellate cells and degraded by hepatic sinusoidal endothelial cells. Levels correlate well with the degree of fibrosis in ALD(Stickel et al., 2003) and HCV(McHutchison et al., 2000). The increase in serum is due to

a combination of reduced hepatic sinusoidal clearance and increased hepatic production (or both).

1.17.2.2 Collagen markers

Several markers fit into this category such as N-terminal procollagen 3 peptide (P3NP) and laminin. Elevated levels of P3NP are observed in various liver diseases such as HCV, ALD and NAFLD(Rosenberg et al., 2004, Nojgaard et al., 2003).

1.17.2.3 Collagenases and their inhibitors

MMP

MMPs are enzymes involved in collagen degradation and tissue remodelling. Levels of TIMP correlate with fibrosis stage(Leroy et al., 2004).

1.17.3 Commercially available biomarker combination tests

Fibrotest: α2-macroglobulin, apolipoprotein A1, haptoglobin, gamma glutamyl transpeptidase and total bilirubin. (Fibromax adds AST, ALT, fasting glucose, total cholesterol and triglyceride to this panel)

Fibrometer: platelets, hyalauronic acid or gamma glutamyl transpeptidase, prothrombin index, AST and α2-macroglobulin.

Hepascore: α2-macroglobulin, hyalauronic acid and gamma glutamyl transferase

Enhanced liver fibrosis (ELF) panel: hyalauronic acid, TIMP-11 and P3NP

Fibrotest™

This model was first described in 2001 in HCV patients(Imbert-Bismut et al., 2001). It is licenced to biopredictive (www.biopredictive.com).

FibroTest™,(Poynard et al., 2007) SteatoTest™ (Poynard et al., 2005)and NashTest™ (Poynard et al., 2006) are three simple blood test panels that have been developed to provide a non-invasive estimate of liver fibrosis, steatosis and steatohepatitis, respectively. The FibroMax ™ (Biopredictive, Paris, France) is the combination of these three blood test panels on the same result sheet and provides researchers and physicians with a simultaneous and complete estimation of liver injury in NAFLD(Morra et al., 2007). The FibroMax™ combines 10 serum markers with the age, sex, height (m) and weight (kg) of each patient;

Fibrotest has been validated in patients with HCV, HBV, ALD and NAFLD in a meta-analysis.(Halfon et al., 2008). AUROC for the diagnosis of bridging fibrosis (F2/F3/F4 vs. F0/F1) was 0.82 *0.83-0.86), 0.831 *0.78-0.83), 0.87 (0.82-0.92) and 0.84 (0.76-0.92) respectively.

The reliability of results has been shown to depend on local laboratory compliance with the pre-analytical and analytical conditions recommended by the quality chart of BioPredictive.

Fibrometer

This was first proposed in 2005 following a study by Cales et al in a viral hepatitis cohort. When compared to ELF, APRI, Forns and Fibrotest it outperformed them(Cales et al., 2005) The same author has also looked at the use of Fibrometer in HIV and HCV co-infected patients (HIV/HCV), noting that diagnostic accuracy in co-infected patients is diminished by the presence of HIV when compared to original studies of mono-infected patients(Cales et

al., 2010). More recently, Boursier showed that a combination of Fibroscan (described below) and Fibrometer gave a diagnostic accuracy of 86.7%; this has been introduced into a sequential algorithm for fibrosis evaluation, which is used in France(Boursier et al., 2012a).

Hepascore

This scoring system was first described in 2005(Adams et al., 2005) in an HCV cohort. A score **>**0.5 provided a specificity and sensitivity of 89% and 63% for significant fibrosis, whereas scores **<**0.5 had 74% specificity and 88% sensitivity for advanced fibrosis. The score was further validated in 2009. Again an HCV cohort was used(Becker et al., 2009). A cutoff score of >0.55 was best for predicting significant fibrosis, with a sensitivity and specificity of 82% and 65% and positive and negative predictive values of 70% and 78%. The ability of Hepascore to predict significant fibrosis (F2–4) as determined by the AUROC was similar in training (0.83) and validation sets (0.81)

ELF

ELF accurately predicts moderate to severe fibrosis in several aetiology in several chronic liver diseases. It was first described in 2004(Rosenberg et al., 2004) and revised in 2009 when it was shown that for chronic HCV the pooled AUROC for prediction of Ishak 4-6 fibrosis was 0.85 (0.81-0.89) (Parkes et al., 2011). In PBC, ELF predicts cirrhosis with an AUROC of 0.76 (0.63-0.89) or significant fibrosis with an AUROC of 0.75 (0.67-0.82).(Mayo et al., 2008) In NAFLD, ELF can predict severe fibrosis, moderate fibrosis and no fibrosis with AUROCs of 0.90, 0.82 and 0.76 respectively.(Guha et al., 2008).

ELF scoring is performed by iQur (http://www.iqur.com/ELFTest.html).

1.18 Imaging

Ultrasound, magnetic resonance imaging (MRI) and computed tomography (CT) have all studied as methods of assessing liver fibrosis. CT and MRI have had some success in the non-invasive evaluation of fibrosis in cardiomyopathy(Nagueh and Mahmarian, 2006) and interstitial lung disease(Lutterbey et al., 2007) leading to their study in liver disease.

Imaging of the liver has evolved rapidly in the last 10-20 years and MRI, high resolution CT and high definition US can all identify changes in the liver parenchyma consistent with cirrhosis. Portal hypertension can be evaluated by the presence of splenomegaly, collateral venous circulation and changes to portal venous flow.

Ultrasound

Ultrasound provides rapid inexpensive information about hepatic architecture. It is widely available. Cirrhotic livers have a typical appearance of nodularity and often show increased echogenicity. Doppler ultrasonography allows operators to gain information about the hepatic vasculature and changes in blood flow associated with portal hypertension or loss of vessel patency. Newer liver imaging techniques such as contrast enhanced ultrasound(Bolondi et al., 2007) are not yet in mainstream use.

Fibroscan (Transient Elastography (TE) /Liver stiffness measurement (LSM))

Currently, Fibroscan is the most commonly used method of assessing liver fibrosis.

A vibration of low frequency and mild amplitude is transmitted into the tissue inducing an elastic shear wave that propagates within the liver. Pulse echo ultrasonic acquisitions then

follow the shear wave and measure its speed. The harder the tissue, the faster the wave propagates. The stiffness of the tissue is then measured in kilopascals.

It is a rapid and reproducible way of assessing liver stiffness, which has the advantage over biopsy of a lower sampling error by virtue of the fact that it assesses a larger area of liver. Unfortunately it is not without problems. Extrahepatic cholestasis, liver injury and inflammation can all affect the reading. Usability is also affected by obesity and small intracostal space both of which can affect the reproducibility and accuracy of readings. It is however excellent at diagnosing liver cirrhosis(Sandrin et al., 2003, Friedrich-Rust et al., 2008b)

The use of two or more non-invasive methods increases the diagnostic accuracy of an individual assay(Castéra et al., 2010b). Recently controlled attenuation parameter (CAP) has been introduced by Echosens® who manufacture the scanners. It is a tool for non-invasive assessment and quantification of steatosis. It works by measuring the attenuation of the ultrasound wave. This corresponds to a decrease in ultrasound wave amplitude. Published data shows it to have an AUROC of 0.91 and 0.95 for the detection of more than 10% or 33% steatosis respectively(Sasso et al., 2010).

Recently LSM values have been shown to correlate with portal hypertension as measured by hepatic venous pressure gradient (HPVG)(Vizzutti et al., 2007). This means that potentially LSM could be used to screen liver patients for portal hypertension without the need for endoscopic surveillance, but is yet to be adopted into mainstream practice. Splenic stiffness has also been measured using TE to predict the presence (but not grade) of oesophageal varices(Stefanescu et al., 2011).

MRI

Several advances have been made recently using MRI. These involve magnetic resonance elastography (MRE)(Huwart et al., 2006), contrast enhanced MRI and diffusion weighted MRI. The advantage of MRE over Fibroscan is that potentially the whole liver can be assessed, there is no acoustic window requirement (rib spaces) and the technique is operator independent. MR is however expensive and time consuming compared to Fibroscan. Diffusion weighted MRI(Taouli et al., 2007)calculates the diffusion of water by quantifying the apparent diffusion coefficient, which is reduced in worsening fibrosis through to cirrhosis.

Magnetic resonance spectrography (MRS) is another use of MRI that allows accurate assessment of hepatic fat content without the need to biopsy(Friedrich-Rust et al., 2010, Banerjee et al., 2013).

1.19 Future Directions

Non-invasive assessment is still in its infancy. Despite many research studies that link current biomarkers to fibrosis stage few have been accepted into routine clinical practice partly because of the lack of a reliable gold standard. Further studies are needed to assess specific patient cohorts with validation cohorts confirming the findings in other centres. Currently non-invasive markers have an unclear role in assessing liver fibrosis but as more is known about the diagnostic accuracy of these markers, the role of liver biopsy to stage fibrosis alone is likely to diminish although it will still be required for diagnosis in many cases.

1.20 Link to research in this thesis

Chapter 2(Corbett et al., 2013) details a study performed in the local hospital (Queen Elizabeth Birmingham) looking at the correlation of LSM and Ishak fibrosis stage. We also used this study to analyse the learning curve required to become competent at scanning.

Chapter 3 is a meta-analysis of common liver fibrosis biomarkers to determine whether they can predict clinical end points, which many would argue, is of more importance than fibrosis which could be viewed as a surrogate end point. Although the markers have all been validated in assessing the degree of liver fibrosis, data on their ability to predict outcomes is less widely available.

Chapter 4 reports the potential of a panel of non-invasive biomarkers to predict fibrosis on liver biopsy. The "Non Invasive Biomarkers of Liver Fibrosis" study (NOBLES) was set up to try and clarify the role of potential new serum liver biomarkers in a prospectively collected cohort of patients attending for liver biopsy. Commonly used indirect markers are assessed as well as the commercially available ones. We have also set out to unearth potential new markers.

CHAPTER 2 DIAGNOSTIC ACCURACY OF TRANSIENT ELASTOGRAPHY IN 'REAL-WORLD' CLINICAL PRACTICE

2.1 Abstract

Background & Aims: The diagnostic accuracy of transient elastography (TE) and the importance of the validity criteria in day-to-day UK clinical practice remains unknown. Our study aims to address this.

Methods: We retrospectively analysed the validity criteria (includes ≥10 successful acquisitions, success rate ≥60% and IQR/median <30%) and accuracy of liver stiffness measurements (LSM) performed by multiple operators under routine clinical conditions at the University Hospital Birmingham, UK (2008-2011).

Results: 2311 LSM were performed. 153 (6.6%) were compared with liver biopsy. The correlation between LSM and modified-Ishak fibrosis stage was superior in patients with a valid LSM (n=97) compared to those with an invalid LSM (n=56) (r_s 0.577 vs. 0.259; p=0.022 and the AUROC for significant fibrosis was greater when LSM was valid (0.83 vs. 0.66; p=0.048). Using an 8 kPa cut-off, the negative predictive value of valid LSM was superior to invalid LSM for the detection of significant (84% vs 71%) and advanced fibrosis (100% vs 93%). Learning curve analysis highlighted that the greatest improvement in LSM validity rates occurs in the operators first 10 LSM, reaching 64.7% validity by 50 LSM.

Conclusion: TE requires minimal training (≥10 observed) and when valid is an accurate tool for excluding advanced liver fibrosis. To ensure the diagnostic accuracy the recommended validity criteria should be adhered to in day-to-day clinical practice.

2.2 Introduction:

Identification of patients with significant liver fibrosis is important to determine prognosis and to aid clinical decision-making, with regards to prioritising therapeutic options and monitoring for disease complications (i.e. HCC, gastro-oesophageal varices). However, due to the fact that most patients with chronic liver disease remain asymptomatic until liver function is compromised, establishing the presence and severity of liver fibrosis remains a clinical challenge. This is further compounded by the problems of liver biopsy, which is widely regarded as a sub-optimal 'gold-standard' due to its invasive nature, sampling error, inter-observer variability and the reluctance of patients to undergo repeat biopsy for disease monitoring(Bravo et al., 2001) Consequently, over the last decade non-invasive, cheaper tools for identifying liver fibrosis have been developed(Rockey and Bissell, 2006), and one in particular transient elastography (TE) (Fibroscan, Echosens; France) has been widely adopted into clinical practice.

TE provides a rapid measurement of liver stiffness by the bedside and has been shown to accurately correlate with histological fibrosis (in particular cirrhosis) in several disease aetiologies(Tsochatzis et al., 2011, Friedrich-Rust et al., 2008b, Talwalkar et al., 2007). Subsequently, TE has gained widespread use as a research tool and in certain specialist centres as a routine clinical tool. The manufacturer recommends that all the following criteria have to be met to consider the liver stiffness measurement (LSM) as valid: 1) ≥10 successful acquisitions, 2) success rate ≥60%, and 3) interquartile range (IQR)/median(M) ratio <0.30(Castéra et al., 2010a). However, the importance of complying with these validity criteria has never been demonstrated in 'real-world' clinical practice, as no study

(retrospective or prospective) to date has shown that obtaining all three of these criteria improves diagnostic accuracy (Lucidarme et al., 2009, Myers et al., 2010). Furthermore, since the introduction of TE in the UK in 2005, there has been a paucity of published data from day-to-day clinical practice.

Between 2008 (first introduced) and 2011, hepatologists at the Queen Elizabeth University Hospital Birmingham (UK) performed 2311 TE examinations as part of the clinical assessment of patients attending the liver outpatients department. Our study has analysed the performance of TE in day-to-day clinical practice in this retrospective cohort and examines the importance of adhering to the recommended LSM validity criteria. In addition, with the use of statistical modelling we evaluated training requirements with regards to operator experience.

2.3 Methods:

2.3.1 Study Population:

Consecutive adult patients, who underwent liver TE as part of a routine liver outpatient visit at the Queen Elizabeth Hospital, Birmingham, UK between August 2008 and July 2011, were retrospectively studied. The decision to perform TE and to refer for a liver biopsy was made on clinical grounds by the specialist hepatologist in clinic (consultant or specialist registrar). Patients with suspected chronic liver disease of any aetiology were included. Institutional Review Board approval was not required.

2.3.2 Liver Stiffness Measurement:

Between the study dates, either a consultant hepatologist or a specialist trainee registrar performed the TE examination during the outpatient clinic visit. In our unit, all operators underwent a certified training session (4 hour) with an Echosens consultant prior to using the M-probe and/or XL-probe in the clinical setting. Prior to certification, the operators performed 3 TE examinations on volunteers with the Echosens consultant in attendance to determine if the application of the TE probe and interpretation of the LSM were correct.

TE was performed using either the M-probe (3.5 Hz frequency) or XL-probe (2.5 Hz frequency) with the Fibroscan® 502 machine (Echosens, France). The manufacturer recommends that the XL probe should be used in patients with a skin-capsular distance >2.5 cm (measured by sonographic imaging). Due to the time constraints in liver clinic, operators

were advised to use the XL-probe in patients with a measured BMI > 30 Kg/m^2 (Myers et al., 2012). In May 2011, our unit began using the Fibroscan® 502 Touch (Echosens, France), which has a built-in automated indicator that recommends the probe best suited to the patient's morphology. In accordance with manufacturer's guidance, all TE examinations are performed in our clinics with the patient lying in the dorsal decubitus position with the right arm extended. The tip of the transducer probe (covered with coupling gel) is placed on the skin in an intercostal space overlying the right lobe of the liver. A time-motion ultrasound image allows the operator to locate a portion of liver at least 6-cm thick and free of large vascular/bony structures. The median value of successful acquisitions (target = 10) is deemed to be representative of the liver stiffness, represented as LSM. If no value was obtained following 10 acquisitions LSM failure was documented. LSM was only classified as 'valid' if all three of the manufacturer's criteria(Castéra et al., 2010a) were met: 1) ≥10 successful acquisitions; 2) success rate was ≥60%; and 3) IQR/M ratio <30%. If any of these three criteria were not met the LSM was classified as 'invalid.'

2.3.3 Liver Biopsy:

Electronic pathology records were reviewed to identify all patients who had a liver biopsy within 12 months of their TE. Histological fibrosis staging was used to assess the accuracy of TE for the diagnosis of significant and advanced fibrosis. In our centre, liver biopsies are routinely reported using the appropriate disease-specific liver fibrosis staging (i.e. Ishak for hepatitis C; Kleiner for non-alcoholic fatty liver disease). For purposes of this study, however, each biopsy was re-assessed independently by two liver pathologists (NM & RB or NM & SH) without knowledge of LSM results or other clinical data. In cases of disagreement,

a consensus was reached by a joint review. To take account of the diverse aetiologies of liver disease, liver fibrosis was staged using a modified version of the Ishak scoring system (Ishak et al., 1995) ,as previously described by Rosenberg et al(Rosenberg et al., 2004) (Table 2-1).

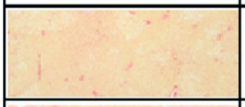
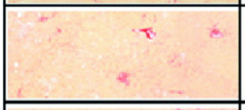
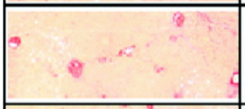
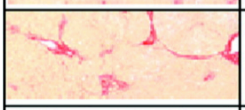
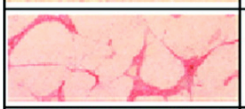
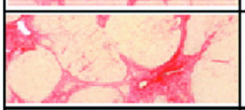
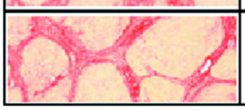
Appearance	Ishak stage: Categorical description	Ishak stage: Categorical assignment	Fibrosis measurement*
	No fibrosis (normal)	0	1.9%
	Fibrous expansion of some portal areas ± short fibrous septa	1	3.0%
	Fibrous expansion of most portal areas ± short fibrous septa	2	3.6%
	Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging	3	6.5%
	Fibrous expansion of portal areas with marked bridging (portal to portal (P-P) as well as portal to central (P-C))	4	13.7%
	Marked bridging (P-P and/or P-C), with occasional nodules (incomplete cirrhosis)	5	24.3%
	Cirrhosis, probable or definite	6	27.8%

Table 2-1 Ishak fibrosis stage. *Proportion (%) of area of illustrated section showing Sirius red staining for collagen (10).

Significant fibrosis was defined as a modified Ishak score > 2 and advanced fibrosis as a modified Ishak score of 5 or 6. The length of biopsy specimens and the number of portal tracts sampled were recorded as measures of biopsy quality. Biopsies specimens that were deemed not adequate by the pathologists for fibrosis staging were excluded from the analysis.

2.3.4 Data collection:

LSM data was retrospectively obtained from all three TE machines in our unit to form a database of the study cohort. Recorded LSM parameters included: patient identification number, date of TE, operator, TE probe, number of successful acquisitions, success rate and median value (IQR) of successful acquisitions. Electronic histopathology reports were reviewed to identify those patients who had an ultrasound-guided liver biopsy within 12 months of the TE examination. Demographics, anthropometric measurements (weight, height, BMI), liver enzymes and liver disease aetiology at the time of TE examination were obtained for these cases. The definitive disease aetiology was determined by a combination of the clinical and histological findings and was categorized into fatty liver disease [non-alcoholic or alcoholic], viral hepatitis [hepatitis B, C], autoimmune [autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis], post-transplant and other, for purposes of statistical analysis.

2.3.5 Statistical Analysis:

The demographics and characteristics of patients were summarised according to the validity criteria of the LSM (*as defined above*). Continuous variables were compared with independent sample t-tests and Mann-Whitney tests (as applicable), and categorical variables were compared with Fisher's exact test.

The strength of the relationship between the LSM and the modified Ishak score was analysed using Spearman's rho correlation coefficients. Separate coefficients were produced for those measurements where each of the three LSM validity criteria were met,

and those where the criteria were contravened. The coefficients were then compared to test whether non-compliance with the LSM validity criteria is detrimental to the ability of TE to predict the histological severity of liver fibrosis. The modified Ishak score was then converted into two binary outcomes indicating the presence of significant fibrosis (Ishak 3-6) and of advanced fibrosis/cirrhosis (Ishak 5 or 6). ROC curves were produced to test the accuracy of LSM in the prediction of significant and advanced fibrosis. Separate ROC curves were produced for LSM that were deemed 'valid' by each of the validity criteria, and those that were 'not valid', with comparisons made between the resulting AUROC. A cut-off value of 8 kPa was used to determine the presence of significant fibrosis, above which further investigation is deemed appropriate(Roulot et al., 2008). Furthermore, the use of different LSM cut-offs to identify patients with significant fibrosis and advanced fibrosis was examined with the calculation of sensitivity and specificity statistics, as well as the PPV and NPV. Analysis of different LSM cut-offs was only performed on LSM that met the validity criteria

Binary logistic regression was used to analyse the effect of the number of TE examinations performed on the likelihood of a valid LSM reading. Prior to the analysis, the scan number was $\log_{10}$ transformed, in order that the model was based on the shape of curve generally observed in a learning curve analysis. The results of the analysis were only reported for the first 100 TE examinations as some operators had performed fewer than 25 scans. This was in order to maximise their usefulness, whilst minimising the amount of extrapolation required. However, all of the data (n=2328) was used in the production of the statistical model.

Analyses were performed using IBM SPSS 19 and Microsoft Excel, with p values less than 0.05 deemed to be indicative of significance.

2.4 Results:

2.4.1 Patient characteristics:

Between 2008 and 2011, 2311 LSM were performed as part of the clinical assessment of patients attending the liver outpatients department. Year-on-year there was an increase in number of LSM performed (2008-2009, 54/month; 2010-2011, 78/month). Of the 2311 LSM, 127 (5.5%) were LSM failures (zero successful acquisitions), 625 (27.0%) were invalid and 1559 (67.5%) were valid LSM (Figure 2-1).

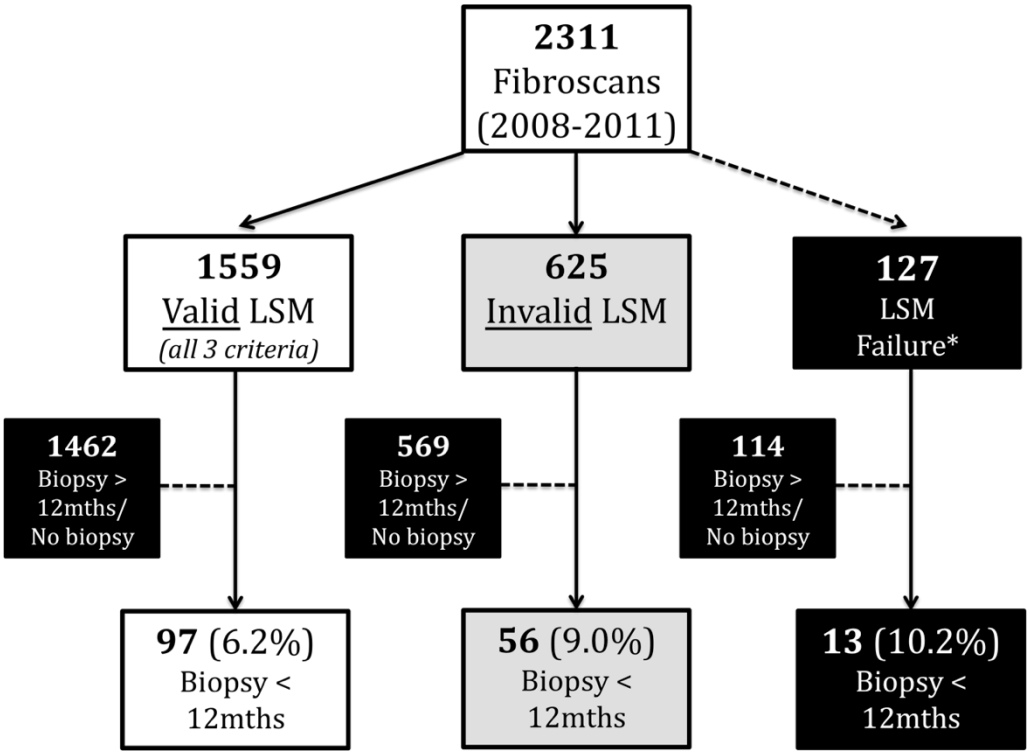


Figure 2-1 Flow diagram of the study.

153 patients were selected after exclusion (*black shading*) of patients in which the operator failed to get a single LSM reading (*defined as LSM failure) and/or when biopsy wasn't performed within 12 months of TE.

	Validity of Transient Elastography		
	Valid (n=97)	**Not Valid (n=56)**	**p-Value**
Characteristics			
Age (Years)[#]	47.4 (1.4)	50.1 (1.5)	0.232
Gender			0.718
- Male	68 (70.1%)	37 (66.1%)	
- Female	29 (29.9%)	19 (33.9%)	
Disease type			0.678
- Fatty liver disease (NAFLD/ALD)	39 (40.2%)	19 (33.9%)	
- Viral (HBV/HCV)	28 (28.9%)	21 (37.5%)	
- Autoimmune (AIH/PSC/PBC)	7 (7.2%)	6 (10.7%)	
- Post-Transplant	10 (10.3%)	5 (8.9%)	
- Other	13 (13.4%)	5 (8.9%)	
BMI [kg/m^2]	27.9 (26.7-29.2)	29.2 (27.3-31.2)	0.263
AST (U/L)	49.9 (44.3-56.3)	48.4 (40.2-58.1)	0.762
Liver Biopsy			
Time difference between biopsy and Fibroscan (days)[†]	70.0 (23.5-122.5)	69.0 (12.8-195.0)	0.953
Portal Tracts (n)	15.1 (13.8-16.6)	14.6 (12.7-16.7)	0.630
Length of biopsy (mm)	15.4 (14.5-16.5)	16.1 (14.7-17.6)	0.477
Modified Ishak Stage of Fibrosis (0-6)			0.387
- 0	14 (14.4%)	10 (17.9%)	
- 1	24 (24.7%)	13 (23.2%)	
- 2	17 (17.5%)	5 (8.9%)	
- 3	18 (18.6%)	9 (16.1%)	
- 4	8 (8.2%)	10 (17.9%)	
- 5	11 (11.3%)	4 (7.1%)	
- 6	5 (5.2%)	5 (8.9%)	
Transient Elastography (TE)			
Operator			0.738
- Consultant	49 (50.5%)	30 (53.6%)	
- Specialist Registrar	48 (49.5%)	26 (46.4%)	
Probe			0.856
- M-probe	68 (70.1%)	38 (67.9%)	
- XL-probe	29 (29.9%)	18 (32.1%)	
LSM (kPa)[†]	9.4 (6.6-14.5)	14.1 (7.3-26.1)	0.011[$]*
LSM per modified Ishak Stage (kPa)[†]			
- 0	5.6 (4.7-6.8)	12.9 (6.8-17.1)	0.008[$]*
- 1-2	8.6 (6.5-10.9)	8.5 (6.1-18.8)	1.000[$]
- 3-4	11.4 (8.7-20.0)	16.0 (8.9-18.6)	1.000[$]
- 5-6	17.3 (12.1-26.0)	48.9 (11.9-68.2)	0.612[$]

Table 2-2 Characteristics of patients who underwent transient elastography + liver biopsy Continuous data displayed as: Geometric Mean (95% Confidence Interval(CI)) Categorical data displayed as: N (%), [#]Data displayed as: Mean (Standard Error(SE)); [†]Data displayed

as: Median (Quartiles), $p-Values Bonferroni-Adjusted for 4 comparisons. Significant* = p<0.05

The mean age of this group was 48.4 (SE 1.1) years, 68.6% were male and the mean BMI was 28.4 Kg/m^2 (95% CI 27.3-29.5). The disease aetiologies (confirmed on biopsy) were fatty liver disease in 37.9% (n=58), viral hepatitis in 32.0% (n=49), autoimmune in 8.5% (n=13), post-transplant in 9.8% (n=15) and miscellaneous/other in 11.8% (n=18).

2.4.2 Liver histology and liver stiffness measurement (LSM):

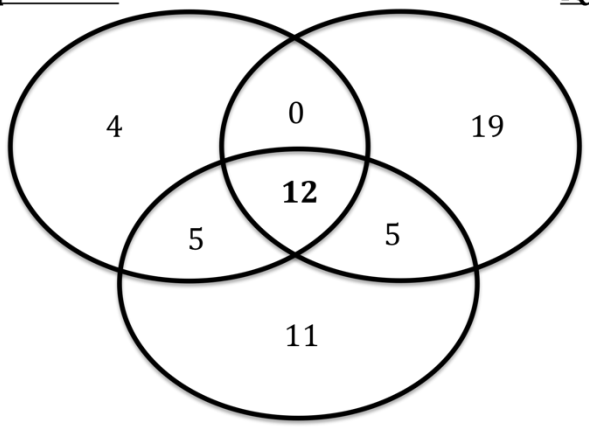
The median time difference between LSM and liver biopsy was 70 days (IQR 22.0-127.0). The mean number of portal tracts and length of biopsy was 14.9 (95% CI 13.9-16.1) and 15.7 (95% CI 14.9-16.5) mm, respectively. The liver pathologists deemed all 153 liver biopsies adequate for fibrosis staging. Seventy patients (45.7%) had significant fibrosis (Ishak stage 3-6), of which 25 had advanced fibrosis (Ishak 5-6). Seventy-eight (51.0%) of the LSM were performed by consultant hepatologists, with remainder by specialist registrars in training. One hundred and six (69.2%) of the LSM were performed using the M-probe versus 47 (30.8%) with the XL-probe. Overall, the median LSM for the population of readings was 10.2 kPa (IQR 6.8-17.1).

2.4.3 Importance of the LSM validity criteria for predictive accuracy:

Of the 153 patients with paired LSM and liver biopsy, 56 (36.6%) patients had an invalid LSM; of whom 21 patients (37.5%) had <10 successful acquisitions, 36 (64.3%) had IQR/M >30%, and 33 (58.9%) had a success rate <60% (Figure 2-2).

< 10 successful acquisitions

Number successful acquisitions	N
1-3	10
4-6	9
7-9	2
Total	**21**



IQR/median ≥30%

IQR/median %	N
30-49	31
50-69	4
70-90	1
Total	**36**

Success rate < 60%

Success rate %	N
1-19	3
20-39	9
40-59	21
Total	**33**

Figure 2-2 Venn diagram highlighting the reason(s) for an invalid LSM.

56/153 patients had an invalid LSM. Tables highlight the distribution of spread for each of the three reasons that the LSM was classed as invalid.

LSM were significantly higher in patients with an invalid scan compared to those with a valid scan (14.1 vs. 9.4 kPa; p=0.011). This was most pronounced in patients without fibrosis on biopsy (12.9 vs. 5.6 kPa; p=0.008). There was no significant difference in age, sex, disease type, BMI, AST and histological parameters between patients with a valid LSM and those with an invalid LSM (Table 1).

The correlation between LSM and modified Ishak fibrosis stage was stronger in patients with a valid LSM compared to those without (r_s 0.577 vs. 0.259; p=0.022) (Figure 2-3).

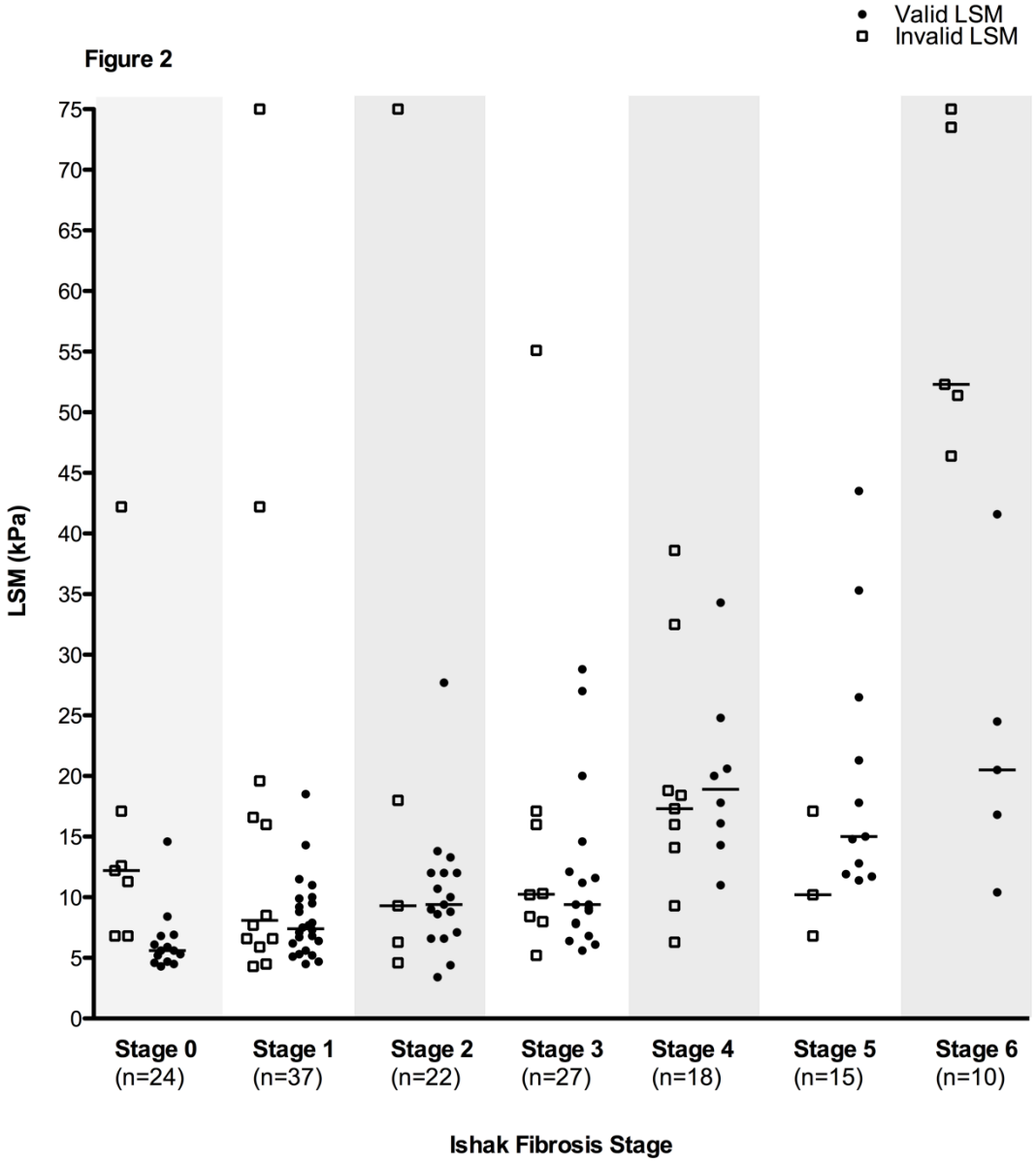


Figure 2-3 Relationship between the LSM and the modified Ishak stage of liver fibrosis for both valid (black circle) and invalid LSM (white box).

Median LSM values for each modified Ishak stage represented by horizontal bar.

The most important individual component of the validity criteria was obtaining a success rate ≥60%. The correlation between LSM and modified Ishak fibrosis stage was significantly greater in patients with success rate ≥60% compared to those with <60% success rate (r_s 0.586 vs. 0.018; p=0.001). This correlation was not significant for IQR/median (≥30% vs.

<30%, r_s 0.520 vs. 0.264; p=0.122) and number of successful acquisitions (≥10 vs. <10, r_s 0.485 vs. 0.350; p=0.514).

2.4.4 Average stiffness values for patients according to Ishak staging

Average stiffness values were calculated for patients with no significant fibrosis (Ishak 1-2), significant fibrosis (Ishak stage ≥3) and cirrhosis (Ishak stage ≥5). The results were then split into those in which all 3 success criteria had been met and those where they were not deemed "unsuccessful". Prediction intervals were also calculated to illustrate the levels of variability in the data.

The results are illustrated in figure 2-4

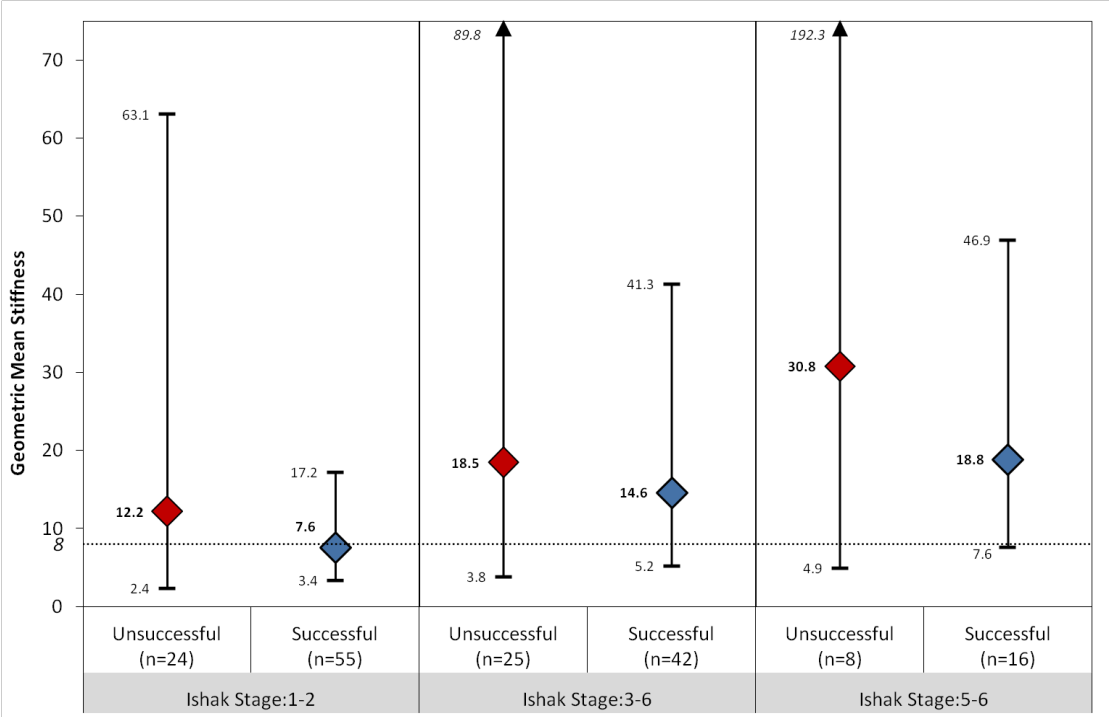


Figure 2-4 Effect of Success on predictive accuracy

Patients with cirrhosis and unsuccessful scans had a lower limit of confidence of 4.9, rising to 7.6 in patients with a successful scan.

2.4.4.1 Ishak>3

Of the 97 valid scans, 68 (70%) agreed with the biopsy (i.e. stiffness≤8 and Ishak<3 or stiffness>8 and Ishak≥3). The effects of a range of factors on the difference between those with and without agreement are tested below:

Factor	***Agreement Between Scan and Biopsy***		**p-Value**
	No	Yes	
BMI	27.7 (25.2-30.3)	28.1 (26.6-29.6)	0.774
AST	47.9 (38.3-59.7)	50.9 (43.9-58.9)	0.647
Portal Tracts (cm)	14.5 (12.5-16.9)	15.4 (13.8-17.3)	0.544
Date Difference (Days)[†]	114.7 (19.9)	84.2 (10.7)	0.183
Diagnosis[#]			0.307
Viral	10 (35.7%)	18 (64.3%)	
Steatohepatitis	10 (25.6%)	29 (74.4%)	
AI/PSC/PBC	0 (0.0%)	7 (100.0%)	
Post-Transplant	4 (40.0%)	6 (60.0%)	
Other	5 (38.5%)	8 (61.5%)	
Operator[#]			1.000
Consultant	15 (31.3%)	33 (68.8%)	
SPR	14 (29.2%)	34 (70.8%)	
Probe[#]			0.029*
M	25 (36.8%)	43 (63.2%)	
XL	4 (13.8%)	25 (86.2%)	

Table 2-3 Factors Predicting Agreement Between Scan and Biopsy

Data displayed as geometric mean (95% confidence interval) and p-values from independent sample t-tests, unless stated otherwise
[#]N(%), with p-value from Fisher's exact test
[†]Median (Quartiles), with p-value from Mann-Whitney test
***Significant at p<0.05**

Hence, it can be concluded that scans performed with the "XL" probe are more likely to be accurate than those performed with the "M" probe (86.2% vs. 63.2%; p=0.029)

2.4.4.2 Ishak>5

Of the 97 valid scans, 54 (56%) agreed with the biopsy (i.e. stiffness≤8 and Ishak<5 or stiffness>8 and Ishak≥5). The effects of a range of factors on the difference between those with and without agreement are tested below:

Factor	***Agreement Between Scan and Biopsy***		**p-Value**
	No	Yes	
BMI	28.5 (26.5-30.6)	27.5 (25.8-29.3)	0.447
AST	52.8 (44.0-63.4)	47.8 (40.5-56.3)	0.410
Portal Tracts (cm)	15.1 (13.2-17.3)	15.2 (13.4-17.2)	0.979
Date Difference (Days)[†]	70.0 (24.0-272.0)	68.5 (5.0-124.5)	0.819
Diagnosis[#]			0.599
Viral	12 (42.9%)	16 (57.1%)	
Steatohepatitis	19 (48.7%)	20 (51.3%)	
AI/PSC/PBC	2 (28.6%)	5 (71.4%)	
Post-Transplant	6 (60.0%)	4 (40.0%)	
Other	4 (30.8%)	9 (69.2%)	
Operator[#]			0.149
Consultant	17 (35.4%)	31 (64.6%)	
SPR	25 (52.1%)	23 (47.9%)	
Probe[#]			0.824
M	31 (45.6%)	37 (54.4%)	
XL	12 (41.4%)	17 (58.6%)	

Table 2-4 Factors Predicting Agreement Between Scan and Biopsy

Data displayed as geometric mean (95% confidence interval) and p-values from independent sample t-tests, unless stated otherwise
[#]N(%), with p-value from Fisher's exact test
[†]Median (Quartiles), with p-value from Mann-Whitney test
***Significant at p<0.05**

Table 2-5 summarises the accuracy of LSM in predicting fibrosis (AUROC), based on obtaining each of the individual validity criteria and when all 3 of the criteria are met (defined as a valid LSM). AUROC for significant fibrosis (Ishak 3-6) was significantly greater when all three of the LSM validity criteria were met (0.83 vs. 0.66; p=0.048), with a success rate ≥60% being the most important individual component to achieve accuracy (Table 2-5a). In contrast, none of the individual components of the LSM validity criteria had a significant effect on the prediction of advanced fibrosis (Ishak 5-6) (Table 2-5b).

Components of the LSM Validity Criteria	AUROC (SE) of LSM based on obtaining individual components of the criteria		p-Value
	No	Yes	
(a) Significant Fibrosis (Ishak 3-6)			
IQR/M <30%	0.75 (0.10)	0.77 (0.04)	0.805
Success Rate ≥60%	0.56 (0.12)	0.84 (0.04)	0.032*
≥10 successful acquisitions	0.58 (0.16)	0.80 (0.04)	0.189
All 3 criteria obtained	**0.66 (0.08)**	**0.83 (0.04)**	**0.048***
(b) Advanced Fibrosis (Ishak 5-6)			
IQR/M <30%	0.69 (0.17)	0.85 (0.04)	0.317
Success Rate ≥60%	0.66 (0.20)	0.86 (0.04)	0.265
≥10 successful acquisitions	0.76 (0.15)	0.82 (0.04)	0.749
All 3 criteria obtained	**0.76 (0.10)**	**0.87 (0.04)**	**0.361**

Table 2-5 Importance of the validity criteria for LSM vs. (a) significant and (b) advanced fibrosis. AUROC of LSM for 153 patients.

Key: Significant* = p<0.05

The published(Roulot et al., 2008, McCorry et al., 2012) LSM cut-off of 8 kPa was used to determine the sensitivity, specificity, NPV and PPV for the presence of significant (Ishak 3-6) and advanced fibrosis (Ishak 5-6) (Table 2-6). A valid LSM produced a sensitivity of 86% (95%

CI 71-95) and specificity of 58% [CI 95% 44–71], whereas an invalid LSM resulted in a sensitivity of 84% (CI 95% 64-95) and a specificity of 42% (CI 95% 22-63) (Table 6a). Subsequently, the NPV for the presence of significant fibrosis was 84% for a valid LSM compared to 71% for an invalid LSM. Furthermore, the NPV for presence of advanced fibrosis was 100% for a valid LSM versus 93% for an invalid LSM (Table 6b).

Components of LSM validity criteria		Sensitivity %	Specificity %	PPV %	NPV %
a. Significant Fibrosis (Ishak 3-6)					
IQR/M <30%	No	88 (64 - 99)	42 (15 - 72)	68 (45 - 86)	71 (29 - 96)
	Yes	84 (71 - 93)	55 (43 - 67)	58 (46 - 70)	82 (68 - 92)
Success Rate ≥60%	No	78 (40 - 97)	29 (10 - 56)	37 (16 - 62)	71 (29 - 96)
	Yes	86 (75 - 94)	60 (46 - 72)	67 (55 - 77)	82 (68 - 92)
≥10 successful acquisitions	No	86 (42 – 100)	29 (4 - 71)	55 (23 - 83)	67 (9 - 99)
	Yes	85 (73 - 93)	56 (43 - 67)	61 (50 - 72)	82 (68 - 91)
All 3 criteria obtained	**No**	**84 (64 - 95)**	**42 (22 - 63)**	**60 (42 - 76)**	**71 (42 - 92)**
	Yes	**86 (71 - 95)**	**58 (44 - 71)**	**61 (47 - 73)**	**84 (69 - 94)**
b. Advanced Fibrosis (Ishak 5-6)					
IQR/M <30%	No	75 (19 - 99)	24 (9 - 45)	14 (3 - 35)	86 (42 - 100)
	Yes	100 (83 - 100)	46 (36 - 57)	28 (18 - 40)	100 (92 - 100)
Success Rate ≥60%	No	67 (9 - 99)	26 (10 - 48)	11 (1 - 33)	86 (42 - 100)
	Yes	100 (84 - 100)	45 (35 - 56)	28 (18 - 40)	100 (92 - 100)
≥10 successful acquisitions	No	100 (29 - 100)	27 (6 - 61)	27 (6 - 61)	100 (29 - 100)
	Yes	95 (76 - 100)	43 (34 - 53)	24 (15 - 35)	98 (89 - 100)
All 3 Criteria obtained	**No**	**88 (47 - 100)**	**32 (18 - 48)**	**20 (8 - 37)**	**93 (66 - 100)**
	Yes	**100 (79 - 100)**	**47 (36 - 58)**	**27 (16 - 40)**	**100 (91 - 100)**

Table 2-6 Importance of the validity criteria for LSM (cut-off > 8 kPa) vs. (a) significant and (b) advanced fibrosis. Sensitivity, Specificity and Positive/Negative Predictive values of LSM for 153 patients.

2.4.5 Optimal LSM cut-offs for the diagnosis of significant/advanced fibrosis

The optimal valid LSM cut-offs in our unit for the diagnosis of significant and advanced fibrosis are outlined in Table 2-7. In general, the LSM cut-off of 10 kPa results in the highest sum of sensitivity and specificity for both significant and advanced fibrosis. With regards to predicting whether a patient has advanced fibrosis (Ishak 5-6), an LSM cut-off of 10 would retain the 100% sensitivity and NPV achieved with a cut-off of 8 kPa, but in addition would increase the specificity and PPV from 47% to 67% and 27% to 37%, respectively.

LSM cut-off (kPa)	Sensitivity	Specificity	PPV	NPV
a. Significant Fibrosis (Ishak 3-6)				
5	100 (92 - 100)	0.15 (0.06 - 0.27)	0.47 (0.37 - 0.58)	1.00 (0.63 - 1.00)
6	98 (87 - 100)	0.31 (0.19 - 0.45)	0.52 (0.40 - 0.63)	0.94 (0.73 - 1.00)
7	90 (77 - 97)	0.47 (0.34 - 0.61)	0.57 (0.44 - 0.69)	0.87 (0.69 - 0.96)
8	0.86 (0.71 - 0.95)	0.58 (0.44 - 0.71)	0.61 (0.47 - 0.73)	0.84 (0.69 - 0.94)
9	0.83 (0.69 - 0.93)	0.67 (0.53 - 0.79)	0.66 (0.52 - 0.78)	0.84 (0.70 - 0.93)
10	**0.74 (0.58 - 0.86)**	**0.78 (0.65 - 0.88)**	**0.72 (0.56 - 0.85)**	**0.80 (0.66 - 0.89)**
11	0.69 (0.53 - 0.82)	0.82 (0.69 - 0.91)	0.74 (0.58 - 0.87)	0.78 (0.65 - 0.87)
12	0.57 (0.41 - 0.72)	0.89 (0.78 - 0.96)	0.80 (0.61 - 0.92)	0.73 (0.61 - 0.83)
13	0.52 (0.36 - 0.68)	0.89 (0.78 - 0.96)	0.79 (0.59 - 0.92)	0.71 (0.59 - 0.81)
14	0.52 (0.36 - 0.68)	0.93 (0.82 - 0.98)	0.85 (0.65 - 0.96)	0.72 (0.60 - 0.82)
15	0.43 (0.28 - 0.59)	0.96 (0.87 - 1.00)	0.90 (0.68 - 0.99)	0.69 (0.57 - 0.79)
20	0.33 (0.20 - 0.50)	0.98 (0.90 - 1.00)	0.93 (0.68 - 1.00)	0.66 (0.55 - 0.76)
25	0.17 (0.07 - 0.31)	0.98 (0.90 - 1.00)	0.88 (0.47 - 1.00)	0.61 (0.50 - 0.71)
30	0.10 (0.03 - 0.23)	1.00 (0.94 - 1.00)	1.00 (0.40 - 1.00)	0.59 (0.48 - 0.69)
b. Advanced Fibrosis (Ishak 5-6)				
5	1.00 (0.79 - 1.00)	0.10 (0.04 - 0.19)	0.18 (0.11 - 0.28)	1.00 (0.63 - 1.00)
6	1.00 (0.79 - 1.00)	0.22 (0.14 - 0.33)	0.20 (0.12 - 0.31)	1.00 (0.81 - 1.00)
7	1.00 (0.79 - 1.00)	0.37 (0.27 - 0.48)	0.24 (0.14 - 0.36)	1.00 (0.88 - 1.00)
8	1.00 (0.79 - 1.00)	0.47 (0.36 - 0.58)	0.27 (0.16 - 0.40)	1.00 (0.91 - 1.00)
9	1.00 (0.79 - 1.00)	0.54 (0.43 - 0.65)	0.30 (0.18 - 0.44)	1.00 (0.92 - 1.00)
10	**1.00 (0.79 - 1.00)**	**0.67 (0.55 - 0.77)**	**0.37 (0.23 - 0.53)**	**1.00 (0.93 - 1.00)**
11	0.94 (0.70 - 1.00)	0.70 (0.59 - 0.80)	0.38 (0.23 - 0.55)	0.98 (0.91 - 1.00)
12	0.75 (0.48 - 0.93)	0.78 (0.67 - 0.86)	0.40 (0.23 - 0.59)	0.94 (0.85 - 0.98)
13	0.69 (0.41 - 0.89)	0.79 (0.69 - 0.87)	0.39 (0.22 - 0.59)	0.93 (0.84 - 0.98)
14	0.69 (0.41 - 0.89)	0.81 (0.71 - 0.89)	0.42 (0.23 - 0.63)	0.93 (0.84 - 0.98)
15	0.56 (0.30 - 0.80)	0.86 (0.77 - 0.93)	0.45 (0.23 - 0.68)	0.91 (0.82 - 0.96)
20	0.44 (0.20 - 0.70)	0.90 (0.81 - 0.96)	0.47 (0.21 - 0.73)	0.89 (0.80 - 0.95)
25	0.25 (0.07 - 0.52)	0.95 (0.88 - 0.99)	0.50 (0.16 - 0.84)	0.87 (0.78 - 0.93)
30	0.19 (0.04 - 0.46)	0.99 (0.93 - 1.00)	0.75 (0.19 - 0.99)	0.86 (0.77 - 0.92)

Table 2-7 Valid LSM vs. significant/advanced fibrosis - Sensitivity, Specificity, Positive/Negative Predictive values of variable LSM cut-offs

Key: Optimal LSM cut-offs **in bold** that maximised the sum of sensitivity and specificity.

2.4.6 Effect of operator experience on obtaining a valid LSM:

In total, 9 consultants and 8 specialist training registrars performed over 25 LSM each and 29 operators performed less than 25 LSM each. The most experienced operator performed 670 LSM, whereas the least experienced performed 1 in clinical practice (excluding the 3 performed during the training day). Binary logistic regression model (Figure 2-5) was used to consider the effect of the number LSM performed on the likelihood of a valid LSM, as determined by obtaining all 3 of the manufacturers validity criteria. The model shows that a 10-fold increase in the number of LSM that an operator has performed significantly improves their odds of obtaining a valid LSM (Odds ratio 1.57, 95% CI 1.39-1.78; $p<0.001$). Figure 3 shows that only 46% of the initial clinical LSM performed by an operator were valid, whereas the validity rate rises to 57% by 10 LSM. After 10 LSM the rate at which the operator achieves a valid LSM slows, reaching 64.7% by 50 LSM and 67.7% by 100 LSM. In order to obtain a valid LSM 80% of the time the model forecasts that approximately 2500 LSM would be required.

The black line represents the model produced from the binary logistic regression analysis. For scans 1-25, the rates of validity across all operators are plotted at each scan number (the red line). Since the number of operators drops off sharply (n=46 to 17 operators) after this point, the subsequent scans are summarized as 9 point moving average, in order to isolate the trend from variability in the data. The model seems to be a reasonable fit to the observed data, suggesting that it is a valid summary of the general trend.

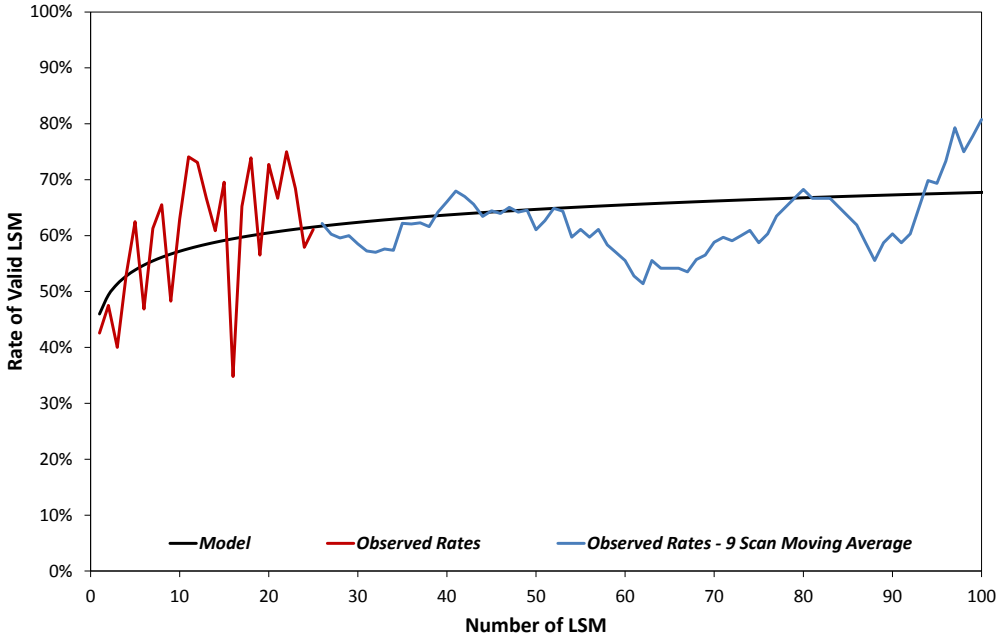


Figure 2-5 Statistical model (learning curve) to highlight the number of LSM that are need to be performed by an operator to achieve a consistent valid LSM.

2.5 Limitations

There was an unavoidable influence of selection-bias that accompanies 'real-world' clinical decision-making that means that some patients may not be represented in this study. The clinician may have chosen to perform a liver biopsy if the high LSM reading was not in keeping with a low pre-test probability of the high result, which may have introduced bias.

As detailed earlier, making a diagnosis of degree of fibrosis based on the flawed gold standard of liver biopsy is a limitation of any study like this. It may be that in future, Fibroscan is considered an alternative gold standard due to its analysis of a greater proportion of liver tissue than liver biopsy.

2.6 Conclusion:

The number of TE being performed in our unit is increasing year-on-year, to over 75 per month. In contrast to previous studies(Lucidarme et al., 2009, Myers et al., 2010, Wong et al., 2010) our 'real-world' data highlights the importance of adhering to the manufacturer's recommended validity criteria (≥10 successful acquisitions, success rate was ≥60% and IQR/M <30%). Adhering to the criteria (and in particular ≥60% success rate) resulted in a significantly greater correlation with liver fibrosis stage and greatly enhanced the accuracy of a negative LSM in ruling out significant (using LSM cut-off > 8 kPa, NPV 84%), and advanced liver fibrosis (NPV 100%). The greatest improvement in ability to achieve a valid LSM occurs in the operators first 10 scans, and thereafter the validity rate progressively increases, albeit very slowly. This study should inform other UK NHS centres that prior to using TE in clinical practice, novices should be trained to understand the clinical implications of the LSM validity criteria and should undertake a minimum of 10 observed scans prior to using TE independently. Our data suggest that failure to meet the LSM validity criteria increases the risk of over interpreting an LSM > 8 kPa and incorrectly labelling a patient as having significant fibrosis, in those without fibrosis (Figure 1). Furthermore, after obtaining an invalid LSM < 8kPa the clinician runs the risk of falsely reassuring 7% patients who have underlying advanced fibrosis (Ishak 5-6). In contrast, when a valid LSM is performed clinicians (consultant or registrar level) can exclude significant fibrosis and to a greater extent advanced fibrosis with a high degree of confidence. In our unit, using the cut-off of 8 kPa with a valid LSM, we could reliably exclude significant and advanced fibrosis (sensitivities Ishak >2 = 86%; Ishak 5-6 = 100% and NPVs Ishak >2 = 84%; Ishak 5-6 = 100%) as effectively as data reported in large prospective studies(Castera et al., 2005, Foucher et al., 2006, Kettaneh et al., 2007), nurse-based studies(McCorry et al., 2012) and recent meta-

analyses(Tsochatzis et al., 2011, Friedrich-Rust et al., 2008b, Talwalkar et al., 2007). In order to reduce the number of false positive LSM in our centre, whilst ultimately maintaining the ability to exclude advanced fibrosis (i.e. NPV 100%), a cut-off of 10 kPa could be adopted. Even by increasing the valid LSM cut-off further our reported number of false positives (LSM over estimates) out-weigh those recently reported in a large 40 study meta-analysis by Tsochatzis et al(Tsochatzis et al., 2011). A possible explanation for the high false positive rate in our study might be the unavoidable influence of selection-bias that accompanies 'real-world' clinical decision-making. For instance, in the event that the clinician has a low clinical suspicion of advanced disease (based on other clinical findings), he/she is more likely to proceed to a liver biopsy in the event of an unexpected high LSM compared to an expected (confirmatory) low LSM. Furthermore, due to time constraints it is routine practice in our centre to use the measured BMI (cut-off 30 kg/m^2 (Myers et al., 2012)) to determine the correct probe to use, rather than measure the skin to capsule distance (as used in previous studies). This may have resulted in the inappropriate use of the M-probe in cases of >2.5cm subcutaneous adipose (despite a BMI < 30 kg/m^2) and therefore overestimates of LSM, as previously reported with the M-probe(Myers et al., 2012). This limitation, however, should be eliminated in future assessment in our centre after the recent introduction of the Fibroscan® 502 Touch, which automatically informs the operator of which size probe to use. As in all studies that utilise liver biopsy to evaluate the performance of TE, inter-observer agreement and sampling error in fibrosis staging must be considered(Boursier and Calès, 2010). In order to minimize these limitations, three liver pathologists (RB, SH, NM) re-staged liver fibrosis and reached a consensus in cases of disagreement (<10% cases). Given that the median time delay between TE and biopsy was 70 days (IQR 22-127) it is unlikely that progression of fibrosis could have contributed to discordance. Furthermore, time delay

between TE and biopsy was not a predictor of false positives/negatives in our study. Due to the sample size of our heterogeneous cohort, the employed LSM cut-offs for significant/advanced fibrosis were generic(Talwalkar et al., 2007, Roulot et al., 2008, McCorry et al., 2012) and not specific to individual disease aetiology and/or probe use. Disease-specific and probe-specific cut-offs still require validation in UK clinical practice.

It is important to understand what defines adequate TE training prior to widespread incorporation into UK clinical practice (including the potential for community-based assessment(McCorry et al., 2012). Previous hospital-based studies have reported contrasting degrees of operator experience that are required to achieve consistent and valid LSM readings (range 20 to >500 LSM required(Castéra et al., 2010a, McCorry et al., 2012, Kettaneh et al., 2007, Boursier et al., 2008). Our statistical model highlights that the initial training period should incorporate a minimum of 10 procedures, with a stable degree of consistency in valid LSM rates by 50 examinations. Furthermore, LSM validity rates were not affected by the grade of the doctor (consultant vs specialist registrar, p=0.738). This is in keeping with previous studies that recommend that a novice, of any medical professional status, can be trained to use TE(McCorry et al., 2012, Boursier et al., 2008). In addition, our study highlights for the first time in clinical practice that complying with the recommended LSM validity criteria (≥10 successful acquisitions, success rate was ≥60% and IQR/M <30%) provides better diagnostic accuracy than those not fulfilling these 3 criteria. For this reason, we would strongly advocate providing guidance on the current LSM validity criteria in the initial training program. Prospective study, however, is required to fully optimize the LSM validity criteria and understand which of the 3 components are key in clinical practice. Due to the well-document methodological challenges of comparing TE to histological fibrosis in

clinical practice(Boursier and Calès, 2010), future studies should focus on the influence of the LSM validity criteria (and modified versions(Boursier et al., 2012b)) on the value of TE in predicting clinical events (i.e. liver failure, HCC, death etc.).

In summary, our study highlights that TE requires minimal training (≥10 observed LSM) and when valid is a useful tool for excluding advanced liver fibrosis in day-to-day UK clinical practice. To ensure the diagnostic accuracy of LSM the manufacturer's validity criteria should be adhered to at all times and incorporated into the training program. Until prospective data are available in UK clinical practice, a positive LSM (≥8 kPa) should be interpreted with care in the absence of stigmata of CLD and the decision to proceed with further investigations and/or monitoring should be influenced by the clinical acumen of the physician.

CHAPTER 3 PROGNOSTIC ACCURACY OF LIVER FIBROSIS BIOMARKERS- A SYSTEMATIC REVIEW AND META-ANALYSIS

3.1 Abstract

Background: Non-invasive biomarkers have been shown to accurately predict significant liver fibrosis but few have been adopted into routine clinical practice

Aim: To investigate whether non-invasive biomarkers are prognostic factors for clinical outcomes (e.g. mortality, cancer) in patients with chronic liver disease.

Methods: A systematic review of studies identified in EMBASE, MEDLINE and Pubmed Central in October 2012 was undertaken by combining terms for clinical outcomes with those of biomarker assessments. Primary studies were included if the prognostic ability of a biomarker had been examined in relation to a clinical outcome in an adult population with liver disease. Where possible, hazard ratios (HRs) for each biomarker were extracted (either directly or from log-rank information) and, if appropriate, pooled across studies using a random effects meta-analysis model.

Results:

The search yielded 1158 articles. 31 screened studies were included in the systematic review. There was substantial clinical and statistical heterogeneity between these studies. Markers assessed were: APRI (13 studies, 7842 patients), Fib-4 (6 studies, 4385 patients) and AST:ALT ratio (6 studies, 1716 patients). Three studies from which hazard ratio (HR) information for overall survival could be extracted were analysed. In patients with HCV an APRI of >1.5-2.0 revealed a summary unadjusted HR of 2.51 (95% CI: 1.37-4.60) for overall mortality and 4.43 (1.64-11.96) for liver-related mortality. Three studies applying Fib-4 (>3.25) to viral hepatitis patients gave a summary unadjusted HR of 3.70 (1.98-6.91) for overall mortality and 6.23 (2.68-14.47) for liver related death.

Conclusion: Use of APRI with a cut off of >1.5-2 and Fib-4 >3.25 provided prognostic value for overall and liver-related mortality in patients with viral hepatitis. Study heterogeneity

restricted further analyses, and further large, prospective, studies are needed to better define the prognostic ability of biomarkers especially in patients with non-viral liver disease.

3.2 Introduction

Liver biopsy is the accepted gold standard for the assessment of liver fibrosis, but its power is reduced by sampling errors and its invasive nature and associated complications preclude its use for frequent monitoring of disease progression (Bravo et al., 2001). This has led to the development of a range of non-invasive biomarkers to identify liver fibrosis and cirrhosis including serum and imaging assessments. Serum markers for the prediction of fibrosis fall into two categories; i) Indirect markers that combine biochemical tests with demographic information (BARD, NAFLD fibrosis score, Fib-4, APRI, AST:ALT ratio) and ii) Candidate fibrosis markers that measure release of peptides into the circulation during fibrogenesis (Martínez et al., 2011). Such markers include ELF (Rosenberg et al., 2004), Fibrotest (Poynard et al., 2004), Forns (Forns et al., 2002) and Hepascore (Adams et al., 2005). In addition, imaging techniques have been developed that measure liver stiffness (i.e. transient elastography), which has been shown to correlate with the extent of liver fibrosis (Shaheen et al., 2007).

The ability of these non-invasive tests to predict liver fibrosis stages varies from study to study and ranges from poor (~0.6) to excellent (>0.95) (Poynard et al., 2007, Friedrich-Rust et al., 2008a, Musso et al., 2011). A major clinical challenge is not only to record fibrosis stage but also to identify those patients at risk of premature death or developing the complications of chronic liver disease. Hyaluronic acid, a key component of the extracellular matrix and P3NP were the first biomarkers used to show a link with the subsequent development of complications of liver disease (Korner et al., 1996, Babbs et al., 1988), Histological staging of fibrosis is a significant predictor of liver-related mortality in patients with HCV (Everhart et al., 2010), and subsequently Poynard et al used liver biopsy as a

comparator in a meta-analysis of the 5-year prognostic value of APRI, Fib-4 and Fibrotest in patients with chronic liver disease (Poynard et al., 2011). They concluded that only Fibrotest (AUROC 0.88) performed similarly to liver biopsy (AUROC 0.86) in predicting survival. However, this study excluded several key biomarkers such as AST:ALT, ELF, Forns, Hepascore and Fibrometer, did not assess liver-related morbidity or mortality, and nor did it assess Hazard Ratios (HR), which quantifies the prognostic effect of a biomarker

The aims of this systematic review were twofold. Firstly to summarise studies that assessed the prognostic ability of non-invasive biomarkers in relation to mortality and liver-related morbidity. Secondly to assess the heterogeneity between studies for each biomarker in relation to clinical and statistical criteria, and to perform a meta-analysis to summarise the prognostic effect of each biomarker in relation to each clinical outcome

3.3 Methods

This systematic review was directed by a protocol registered on the PROSPERO database (http://www.crd.york.ac.uk/PROSPERO) with reference number CRD42012003244. Studies published as full papers were identified on MEDLINE, PubMed Central and EMBASE using text terms. Studies with only an abstract were excluded. Terms relating to clinical outcomes (e.g. 'encephalopathy' OR 'death' OR 'liver transplant' OR 'mortality' 'ascites' OR 'cancer' OR 'variceal' OR ''varices') were combined with an AND operator with terms relating to biomarker assessment (e.g. 'Aspartate Platelet Ratio Index' (APRI), OR 'Fib-4,' OR 'aspartate-to-alanine transaminase ratio' (AST:ALT), OR 'BARD,' OR 'NAFLD Fibrosis Score,' OR 'Enhanced Liver Fibrosis' (ELF) OR 'Hepascore,' OR 'Fibrotest,' OR 'Fibrometer,' OR 'Forns' OR 'Fibroscan,' OR 'transient elastography'). The biomarkers chosen were those that were acknowledged as being validated for the prediction of onset and diagnosis of liver fibrosis. Searches were conducted in October 2012, and given the paucity of biomarker studies in the previous ten years searches were restricted to those published after 1 January 2002. Titles and abstracts of the search results were screened for relevance. Full texts of relevant articles were obtained and assessed for inclusion in the review against the following criteria: Patients >18 years old and that the biomarker in question was used for the prognosis of a clinical endpoint rather than the diagnosis of its presence. Both stages of the selection process were undertaken independently by two reviewers (CC and MJA) and disagreements were resolved by discussion. This systematic review was reported in accordance with the PRISMA guidelines (www.prisma-statement.org).

3.3.1 Data collection:

Data were extracted from study reports independently by two authors (C.C. and M.J.A) using a prepared checklist. Discrepancies were resolved by discussion involving a third reviewer (P.N.) if necessary. The following data were extracted from each study: year of study, disease type, clinical outcome measures, non-invasive biomarkers used, biomarker cut-off(s), number of patients, and the reported measure of prognostic effect, for example Area under the ROC curve (c-statistic), HR, or relative risk (RR) for each clinical outcome (*listed in the search criteria*). Where prognostic effects were poorly reported we sought to derive the HR estimate and its confidence interval (CI) indirectly using the methods of Parmar et al(Parmar et al., 1998) by examining other available data such as log rank test statistics, their p values, and total number of patients and events in each group. Both unadjusted and adjusted HR estimates were sought for each study, and the choice of adjustment factors recorded for each study. Quality assessment of studies included was performed in accordance with QUADAS-2 guidelines for diagnostic test studies (supplementary table 1) modified with prognostic rather than diagnostic components, as the aim was to assess the prognostic ability of each marker. When assessing patient selection, risk of bias was assessed by looking at patient enrolment and study design. Here we also made sure that the included patients matched the review question. When assessing the index test, we assed if the test was interpreted without knowledge of the reference standard, and also if a threshold was used. The reference standard in our question was a clinical outcome, but we also assessed if the standard was assessed without knowledge of the index test result. Finally, flow and timing of the studies were assessed by determining how many patients were excluded and that they were all assessed against the same outcome.

3.3.2 Statistical analysis:

Meta-analyses of prognostic results were considered for each test in relation to each outcome separately, if HR estimates and their 95% confidence intervals could be obtained for at least two studies. Studies were not qualitatively summarised if the cut-offs varied too much. Cut-offs were also not summarised if they were analysed as a continuous variable or if data were mixed between univariable (unadjusted) and multivariable (adjusted) reporting. Studies were also excluded if they compared different cut offs within the same study (e.g. comparing an AST:ALT of 1-2 vs. 2). Where meta-analysis was appropriate, interest effect estimates for each test were pooled across trials using a random effects meta-analysis model within STATA (version 12). This model accounts for between-study heterogeneity in the log HR effect estimates, which is likely to occur. Heterogeneity across studies was examined using the I-squared statistic, which gives the percentage of the total variability in the data due to between-study heterogeneity. Each random-effects analysis was summarised by reporting the summary (average) prognostic effect estimate and its confidence interval. Also a 95% prediction interval was calculated, to reveal how the prognostic effect of the marker may vary in different contexts and populations, due to the unexplained heterogeneity. Due to the small number of studies suitable for inclusion in the meta-analyses (<10 studies), examinations of subgroups, meta-regression and funnel plot asymmetry (potential publication bias) were not considered sensible as per current guidance(Sterne et al., 2011).

3.4 Results

The literature search identified 1456 studies and after removal of duplicates 1158 were screened for relevance to the review. 100 full articles were subsequently obtained and assessed against the full selection criteria resulting in 31 studies (reported in 31 papers) being included. The majority of studies (n=59) were excluded on the basis that the biomarker was used for diagnosis of the disease rather than examining the prognostic association of the marker with future clinical outcomes in individuals with the disease.

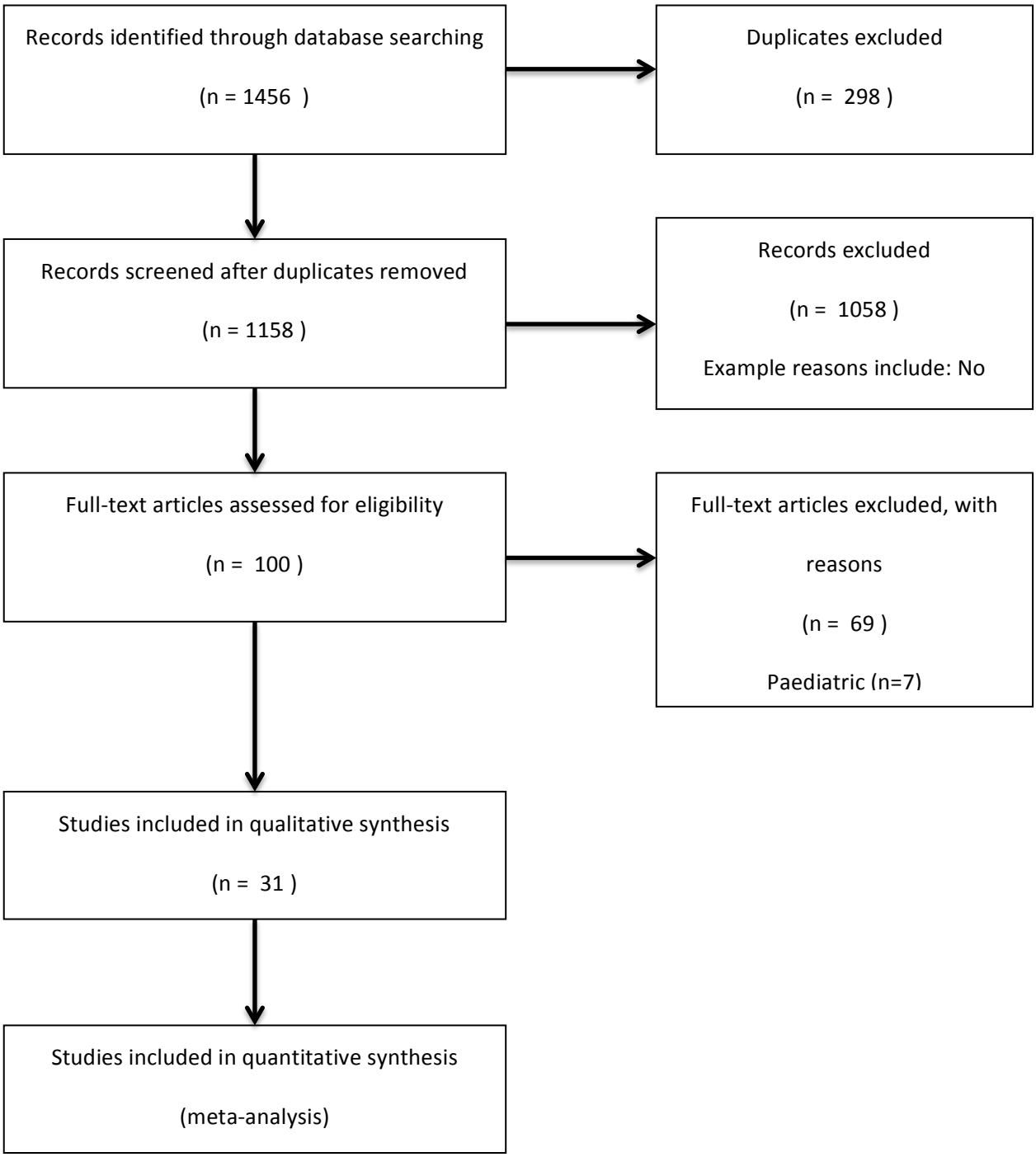


Figure 3-1Flow diagram of study inclusion

3.4.1 Methodological differences:

When grouping studies for potential meta-analysis of each biomarker, there was considerable between-study heterogeneity in the analysis method (continuous vs. categorical modelling of the candidate prognostic factor), chosen cut-off levels, the groups of outcomes recorded, follow up duration and population of patients at baseline. The most commonly analysed biomarkers were the indirect markers and they were mainly examined in patients with viral hepatitis. Characteristics of the included studies are summarised in table 3-1.

3.4.2 Quality assessment

All studies were well designed in terms of patient selection, but it was not possible in some cases to assess if the biomarker was measured without knowledge of the outcome measure. This would have been the case where stored blood samples were analysed after the event in retrospective studies.

				follow up (months)	Continuous if not defined		All death	Liver death	Complications	All death	Liver death	Complications
Bambha 2012	HIV/HCV	P	450	6.6 years	APRI>1.5	All cause mortality compared to <0.5	2.78 (1.87- 4.12) *					
					Fib4>3.25	All cause mortality compared to <1.5	2.58 (1.68-3.95) *					
Chon 201	HBV	P	1126	NK	APRI	HCC or Hepatic decompensation and or HCC						0.73
					Fib4							0.74
					LSM							0.79
Fung 2011	HBV	P	528	35	LSM >10 kPa	HCC, liver related mortality		4% v 0% p<0.001	9% v 0% p<0.001			
Ghany 2011	HCV	P	470	6.3 years	AST:ALT ≤0.8 vs. >0.8	Liver deaths or transplant		0.558 (0.342-0.91)				
Jain 201	HIV± HBV/HCV	P	151	75.3	APRI>1.5	Overall survival	2.79 (1.32-5.91)					
					FIB-4>3.25		3.83 (1.68-8.77)					
Kim 2012	HBV	P	128	27.8	LSM	Liver related outcomes (decompensation and HCC)			1.038 (1.002-1.081)*			
					LSM ≥19kPa				7.176 (2.257-22.812)			
Merchante 2012	HIV+HCV	P	239	20.7	APRI>1.5	Hepatic decompensation ±HCC, liver related mortality or transplant, death of any cause or transplant	1.07 (0.4-2.86)	0.92 (0.27-3.2)				
					Fib-4>3.25		1.68 (0.63-4.48)					
					LSM>40 kPa		0.9 (0.8-1.1)* cont variable	1.03 (0.98-1.07)* cont variable	1.03 (1.01-1.05)* cont variable	0.60	0.73	0.72
Ngo 2008	HBV±HIV	P	978	7.7 years (av)	Fibrotest	Overall survival, survival without HCV complications, survival without HCV death				0.94	0.95	0.89
					APRI					0.57	0.58	0.55
Ngo 2006	HCV	P	260	NK	APRI	Survival without HCV complications or death. Overall survival				0.67	0.76	0.82
			537		Fibrotest					0.76	0.96	0.96
			170		Forns					0.73	0.87	0.86
Nunes 2010	HCV±HIV	P	303	61.8	APRI>1.5	Liver related death then split into 1,3 and 5 year liver related mortality (5yr shown)		10.18 (4.86-21.32)			0.85	
					AST:ALT>1			4.82 (2.35-9.90)			0.72	
					Fib-4>3.25			9.45 (4.51-21.32)			0.85	
Sanvisens 2011	HCV ±HIV IVDUs	P	497	92.4	Fib-4>3.25	Death: advanced liver fibrosis v no liver fibrosis	3.89 (2.28-6.64) RR					
					Forns>6.9		2.96 (1.7-5.15) RR					
Sinn 2008	HCV with treatment	P	1137	55.2	APRI>1	Disease progression			10.5 (2.4-46.6)			
	HCV without treament								5.4 (3.5-8.3)			
Vergniol 2011	HCV	P	1397	47.3	APRI>2	Overall survival and survival without liver related death	3.5 (2.6-4.8)	5.9 (3.9-9)		0.66	0.69	
			1401		Fib-4>3.25		5.3 (3.9-7.2)	9.6 (6-15)		0.75	0.76	
			1414		Fibrotest>0.74		209 (70-626)	1598 (246-1000)		0.8	0.81	
			1457		LSM>9.5kPa		48 930-810	143 (66-309)		0.82	0.87	
Yu 2006	HCV treated at 6 months	P	776		APRI>1.5	All cause mortality, HCC and all liver related outcomes				0.87		0.88
	HCV untreated at baseline		562							0.53		0.72
Bhala 2011	NAFLD	P	247	85.6 (av)	AST:ALT >1	Overall mortality	3.74 (1.1-12.68)					
Changchien 2008	HCC	R	6381	NK	AST:ALT 1-2	Survival	1.260 (1.166-1.361)*					
					AST:ALT >2		1.556 (1.419-1.706)*					
Chen 2006	HCC	R	11312	NK	AST:ALT 1-2 v<1	Overall survival	1.614 (1.53-1.702)					
					AST:ALT >2 v <1		3.261 (3.08-3.453)					

Haukeland 2008	ALD	R	170		AST:ALT	Increase in mortality for every 0.1 rise in ratio	1.03 (1.01-1.04)					
Hino 2003	AIH	P	73	70.2 (av)	AST:ALT	Overall mortality	11.67 (1.15-118.66)					
Ichikawa 2009	HCC	P	366	NK	APRI >10 / cont.	Posoperative hepatic failure			1.125 (1.045- 1.211) OR			
Kao 2011	HCC	R	190	30.7	APRI>1	Overall survival	2.36 (1.24-6.34) 2.53 (1.20-5.32) *					
Klibansky 2012	Mixed	P	667	861 days	APRI>1.5 compared to <0.5	Liver related outcomes such as death, first variceal bleed, HCC, listed for transplant.			21.97 (5.17-93.31) Rel Hazard			0.87
					AST:ALT				1.61 (1.21-2.16) Rel hazard			
					LSM >12.5 kPa comp to <10.5							
Koch 2011	ITU patients	P	108	NK	LSM>18 on admission to ITU	ITU survival and long term survival	Log rank 4.96					
							Log rank 7.06					
Lindvig 2012	Medical admissions	P	212	30	LSM>8					0.80		
Mayo 2008	PBC	P	161	7.3 yrs	ELF measured prior to event	Clinical progression- new varices, variceal bleed, liver related death or transplant.						0.748 0 yr, 0.784 8 yr
Naveau 2009	ALD	P	218	NK	APRI					0.56	0.59	
					Fib-4					0.64	0.65	
					Fibrotest					0.69	0.79	
					Forns					0.43		
					Hepascore					0.69	0.80	
Nyblom 2006	PBC	R	126	NK	AST:ALT	Overall mortality	Not sig					
Nyblom 2007	PSC	R	72	NK	AST:ALT>1	Liver related death		3.82 (2.2-6.7)				
Parkes 2010	Mixed	P	457	7 years	ELF continuous	Liver related outcomes (death and morbidity)			2 (1.8-2.2)			0.86
					ELF 8.34-10.425				6.1 (1.8-20.6)			
					ELF 10.426-12.51				36.4 (10.9-120.9)			
					ELF 12.52-16.67				115.9 (33.2-405.1)			
Sporea 2011	Mixed	P	1000	NK	LSM>50.7	Occurrence of variceal bleeding						0.73
Treeprasertuk 2010	PSC	P	150	NK	AST:ALT	Liver related outcomes			Not sig			

Table 3-1 List of papers included with study characteristics and AUROC values where quoted.

3.4.3 APRI systematic review

Thirteen studies (total patients 7842) used APRI either as a continuous variable or with varying cut offs from >1 to >2. Median follow up ranged from 20 to 75.3 months

3.4.3.1 HCV:

Using APRI as a continuous variable Ngo et al (Ngo et al., 2006) examined 260 patients and found an AUROC for overall survival of 0.67, whilst AUROCs for overall survival excluding HCV-related death and complications were 0.76 and 0.82 respectively. When APRI was used with a cut off (Yu et al., 2006) AUROCs for all-cause mortality varied as expected on the cut-off value used and also on its application before or after anti-viral treatment. AUROC values using an APRI cut off of >1.5 were lower (0.531) in untreated patients at baseline than for patients 6 months after anti-viral treatment (0.875). Furthermore, measurement of the APRI score 6 months after treatment also had a good discriminatory value for the development of HCC with an AUROC of 0.870 during a mean follow up of 4.75 (1-12.2) years. This compared favourably to its use in the untreated cohort where an AUROC of 0.715 was observed. When liver-related deaths were examined using a cut off of >0.75, the relative risk (RR) was 5.78 (2.31-14.45) in the treated cohort. Use of an APRI cut-off of >1 in treated and untreated cohorts of patients with viral hepatitis demonstrated that APRI predicted the development of liver related outcomes, although AUROC statistics were not provided (Sinn et al., 2008). However, multivariate analysis for liver related outcomes of the untreated cohort revealed a HR of 5.4 (3.5-8.3) rising to 10.5 (2.4-46.6) in the treated cohort (Sinn et al., 2008). In a separate study of nearly 1500 patients with viral hepatitis the HR for overall prognosis was 3.5 (2.6-4.8) when using a cut-off of >2 for APRI (Vergniol et al., 2011).

3.4.3.2 HCV ± HIV:

Three studies used an APRI cut-off >1.5 to assess its prognostic value for overall or liver-related mortality. Merchante et al found no significant prognostic value (Merchante et al., 2012), whereas Bambha et al quoted an HR for all-cause mortality of 2.78 (1.87-4.12) when compared to patients with an APRI cut-off of <0.5 (Bambha et al., 2012). Nunes et al provided AUROC data for the ability of an APRI cut-off of >1.5 at three time points to discriminate between those without and those with a definite or probable liver-related mortality (0.9 at 1 year, 0.88 at 3 years and 0.85 at 5 years). The reported HR for liver-related mortality of individuals with an APRI >1.5 (vs. <1.5) was 10.18 (4.86-21.32) (Nunes et al., 2010a).

3.4.3.3 HIV± HBV or HCV:

Only one study (n=151) looked at this cohort (Jain et al., 2012), and with an APRI cut-off of >1.5 the HR for overall survival was 2.79 (1.32-5.91) and the HR for adjusted all-cause mortality was 2.64 (1.22-5.75).

3.4.3.4 HBV:

One study of 1126 patients looked at the use of APRI as a continuous variable in an HBV cohort with emphasis on the development of HCC (Chon et al., 2012). Its AUROC was 0.729 (0.659-0.799) and when expanded to include patients developing hepatic decompensation the AUROC rose to 0.787 (0.705-0.869).

3.4.3.5 Hepatocellular carcinoma:

In a study of patients following radio-frequency ablation (RFA) for HCC (Kao et al., 2011), an APRI cut-off of >1 (vs. ≤1) gave an adjusted HR for overall survival of 2.528 (1.201-5.320). HCC recurrence and development of metastases were also associated with APRI using the same cut off with an adjusted HR of 1.877 (1.25-2.82) and 2.143 (1.172-3.919) respectively. Ichikawa et al used two different cut-offs in a cohort of patients who were to undergo resection for HCC (Ichikawa et al., 2009). APRI as a continuous variable was an independent pre-operative risk factor for postoperative hepatic failure (Odds Ratio (OR)=1.098 (1.018-1.184)). The authors compared patients with low vs. high APRI (<10 and ≥10) and found that patients with a high APRI had an increased incidence of ascites, pleural effusion and death from hepatic failure.

3.4.3.6 Other aetiologies:

Two studies looked at APRI in patients with a non-viral cause for their liver disease and found mixed results. In a cohort of 667 patients with mixed liver aetiologies (Klibansky et al., 2012), the univariate relative hazard for liver related outcomes was 21.97 (5.17-93.31) using an APRI cut-off of >1.5. In contrast, for patients with ALD the prognostic strength was much less with an AUROC for overall death of 0.56 (0.48-0.64) (Naveau et al., 2009). When non-liver related death was examined, the AUROC rose only slightly to 0.60 (0.50-0.69).

3.4.4 AST:ALT ratio systematic review

Six studies were identified that included a total of 1716 patients of differing aetiologies, with follow-up ranging from 29 to 94 months.

3.4.4.1 Immune-mediated liver disease:

For patients with PBC the AST:ALT ratio was not associated with the development of any clinical outcomes (Nyblom et al., 2006). Two papers were identified in cohorts of patients with PSC (Treeprasertsuk et al., 2010, Nyblom et al., 2007), and using AST:ALT ratio as a continuous variable, no prognostic ability was seen. However, when a cut off of >1 was used, a HR of 3.82 (2.2-6.7) was seen for liver-related death (median follow up 8 years; range 1-28 years). A small study of 73 patients with autoimmune hepatitis (mean follow-up 70.2 months) using AST:ALT ratio as a continuous variable, found a univariate HR for death of 3.25 (CI not quoted) with an OR of 11.67 (1.15-118.66) (Hino et al., 2003).

3.4.4.2 HCV:

Using an AST:ALT ratio of <0.8 vs. >0.8 as a prognostic factor for hepatic decompensation produced an HR of 0.416 (0.233-0.743) and a HR 0.558 (0.342-0.910) for liver related deaths or liver transplant (Ghany et al., 2011). When an AST:ALT ratio of >1 was used the AUROC for liver-related death at 5 years in another study was 0.72 (0.61-0.80) (Nunes et al., 2010a).

3.4.4.3 Other causes of liver disease:

In a cohort of 247 NAFLD patients with a mean follow up of 86.5 months an AST:ALT ratio of >1 was associated with an adjusted OR for overall mortality of 3.74 (1.1-12.68) (Bhala et al., 2011). An increase of 0.1 in the ratio resulted in an increased mortality with an HR of 1.07 (1.04-1.1) in a small (n=170) Norwegian cohort of ALD patients (Haukeland et al., 2008). In a mixed liver disease cohort using the AST:ALT ratio as a continuous variable, a HR of 1.61

(1.21-2.16) was described, but this was not found to be significant after multivariate analysis (Klibansky et al., 2012).

3.4.4.4 Hepatocellular carcinoma:

In patients with HCC, only two papers were found that used the AST:ALT ratio. Chen et al (Chen et al., 2006) quoted two cut-offs; if the ratio was 1-2 then the OR of overall survival was 1.623 (1.535-1.716), however if the ratio was increased to >2, the OR rose to 2.969 (2.792-3.158). Similar results were also found by Changchien et al with a HR for survival of 1.260 (1.166-1.361) and 1.556 (1.419-1.706) using AST:ALT ratios of 1-2 and >2 respectively (Changchien et al., 2008).

3.4.5 Fib-4

Six studies using Fib-4 were identified that included a total of 4385 patients and were split between those using it as a continuous variable and those that chose a cut-off of >3.25. Most of the patients in these studies had viral liver disease.

3.4.5.1 Viral liver disease:

Using a cut-off of >3.25 in a cohort of patients with HCV Vergniol et al (Vergniol et al., 2011) showed that the HR for overall survival was 5.3 (3.9-7.2) and 9.6 (6-15) for survival without a liver-related death. In cohorts including patients with HCV who were co-infected with HIV or HBV, the AUROC for prognosis was 0.85 (0.76-0.90) at 5 years whilst the HR for prognosis was 9.45 (4.51-19.79) (Nunes et al., 2010a). Two further studies generated HR values of 2.58 (1.68-3.95) when comparing Fib-4 cut-offs of >3.25 to <1.5 (Bambha et al., 2012) and 3.83

(1.68-8.77) using >3.25 compared to <3.25 as the cut-off (Jain et al., 2012). A study of drug users with advanced liver fibrosis (Sanvisens et al., 2011) found that a Fib-4 cut-off of >3.25 was associated with a RR of death of 3.89 (2.28-6.64), whilst Merchante et al (Merchante et al., 2012) did not identify significance for a Fib-4 cut-off of >3.25 in an HIV / HCV co-infected cohort. Using Fib-4 as a continuous variable in a cohort of patients with HBV an AUROC of 0.744 (0.676-0.813) was found which increased to 0.784 (0.683-0.886) if hepatic decompensation was included as an outcome (Chon et al., 2012).

3.4.5.2 Alcohol induced liver disease:

One study of 218 patients looked at Fib-4 (cut-off unclear) in this cohort (Naveau et al., 2009), identifying an AUROC for overall death of 0.64 (0.55-0.71).

3.4.6 ELF panel

In 2008, Mayo et al (Mayo et al., 2008) prospectively analysed 161 PBC patients and concluded that ELF could be a prognostic factor for clinical outcomes related to liver disease (e.g. development of new varices, variceal haemorrhage, ascites, encephalopathy, liver related death or liver transplant). Each 1-point increase in ELF was associated with a threefold increase in future complications. In addition, they found that 6 years prior to the first complication, ELF had a prognostic AUROC of 0.684 (0.528-0.814). In 2010, with larger numbers of patients, a broader range of liver aetiologies, and using ELF as a continuous variable (Parkes et al., 2010) it had an OR of 2.2 (1.7-2.9) for prognosis of liver outcomes 6 years later. If ELF was used with a higher cut-off (12.52-16.67) rather than being analysed as

a continuous variable, the OR rose to 75.7(17.6-325.4) with an AUROC of 0.86 (0.79-0.93) at 6 years.

3.4.7 Fibrotest

Ngo et al published 2 papers studying Fibrotest as a continuous variable in groups of patients with differing aetiologies. In a study of 1074 HBV (±HIV) patients there was an AUROC for overall survival of 0.94 (0.89-0.96), whilst for survival without liver complication or death, the multivariate HR was 5.21 (3.53-6.88) (Ngo et al., 2008). In a cohort of 537 patients with HCV there was an AUROC for the prognosis of overall survival of 0.76 (0.63-0.84) and 0.96 (0.93-0.98) for survival without death from liver disease (Ngo et al., 2006). Vergniol et al (Vergniol et al., 2011) also looked at patients with HCV, but used a Fibrotest cut off of >0.74 and showed a HR for overall survival of 209 (70-626). When used in a cohort of 218 patients with ALD, the AUROC for overall death was 0.69 (0.61-0.76) giving a risk ratio (RR) for overall death of 3.7 (1.2-11.7) and 23.2 (3.2-167.3) for liver-related death (Naveau et al., 2009).

3.4.8 Forns

Three studies looked at the prognostic value of Forns. In patients with HCV (±HIV) and advanced liver fibrosis, Sanvisens (Sanvisens et al., 2011) used a cut off of 0.74 and found a RR of death of 2.96 (1.7-5.15) when comparing them to patients without advanced liver failure. Another study in patients with HCV demonstrated the AUROC for overall survival was 0.73 (0.46-0.87), which rose to 0.87 (0.52-0.97) for survival without liver-related death or 0.86 (0.74-0.93) for survival without complications of liver disease (Ngo et al., 2006). In a

cohort of patients with ALD (Naveau et al., 2009), the AUROC for prognosis of overall death was 0.43 (0.35-0.51).

3.4.9 Hepascore

Only one paper looked at Hepascore for its prognostic value; Naveau et al (Naveau et al., 2009) analysed data in a cohort of patients with ALD using Hepascore as a continuous variable, and demonstrated an AUROC for prognosis of 0.69 (0.62-0.76) with a RR for death of 0.9 (0.2-3.1).

3.4.10 LSM

There have been several studies looking at LSM and its prognostic value, but comparison was not possible due to marked heterogeneity of disease types, liver stiffness cut-offs and clinical outcomes. Disease types included HBV (n=3), HCV (n=2), mixed liver aetiology studies (n=2) and acute/intensive medical illness (n=2). The range of LSM cut-offs (kPa) ranged from >8.0 up to 50.7 kPa (Fung et al., 2011, Chon et al., 2012, Kim et al., 2012b, Merchante et al., 2012, Vergniol et al., 2011, Lindvig et al., 2012, Koch et al., 2011, Klibansky et al., 2012, Sporea et al., 2011). Details of these studies are summarised in table 3-1.

3.5 Meta-analysis

3.5.1 Random effects meta-analysis of HCV and APRI to predict overall death:

Meta-analysis of three studies (Vergniol et al., 2011, Merchante et al., 2012, Jain et al., 2012) from which unadjusted HR estimates for overall death could be extracted was performed. The summary HR for an APRI cut-off of >2 (Vergniol et al., 2011) was 3.5 (95% CI: 2.58-4.76), whereas for an APRI cut-off of >1.5 (Merchante et al., 2012, Jain et al., 2012) the HR was 1.82 (95% CI: 0.71-4.65). An overall summary HR of 2.51 (1.37-4.60) for APRI >1.5-2 was calculated as illustrated in Figure 3-2, with a high proportion of between study heterogeneity (I-squared 81.2%).

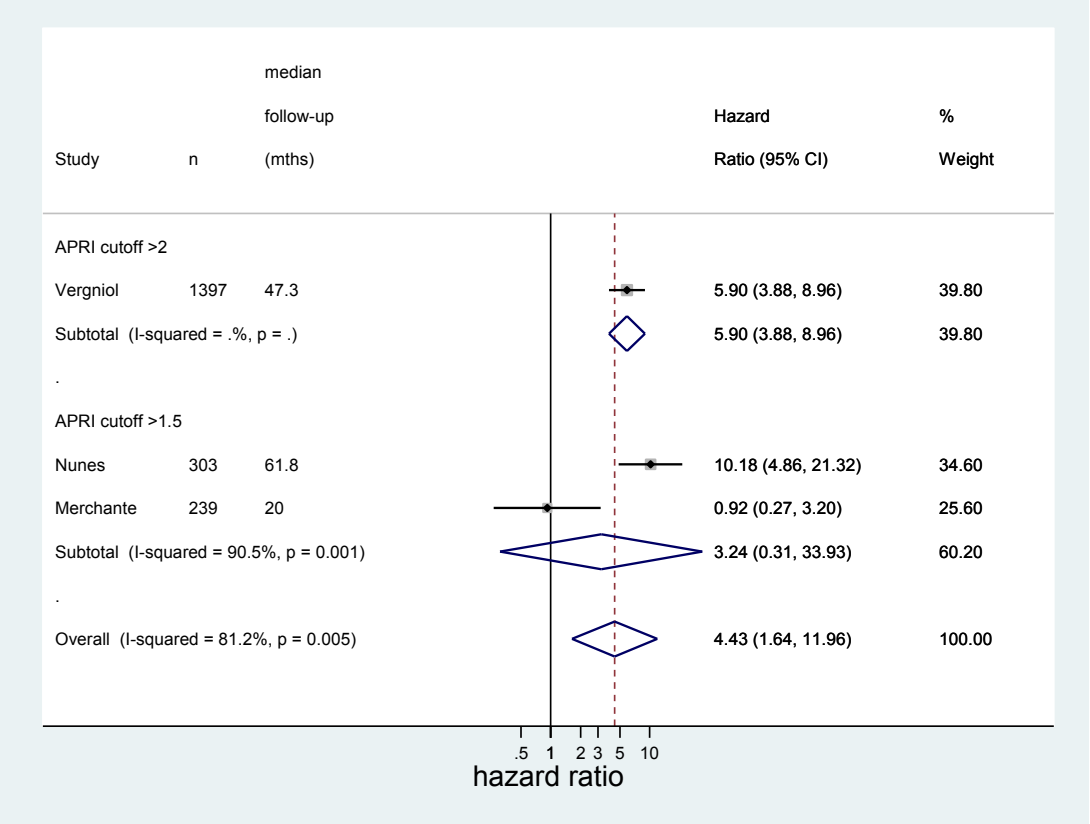


Figure 3-2 Meta-analysis of APRI to predict overall death in Hepatitis C patients

3.5.2 Random effects meta-analysis of HCV and APRI to predict liver death:

Analysis of 3 studies (Vergniol et al., 2011, Nunes et al., 2010a, Merchante et al., 2012) was possible using the criteria indicated earlier. The HR for an APRI cut-off>2 was 5.90 (2.88-8.96), whereas for an APRI cut-off of >1.5 the HR was 3.24 (0.31-33.93). An overall unadjusted summary HR of 4.43 (1.64-11.96) for an APRI >1.5-2 was calculated as illustrated in Figure 3-3. Again the between study heterogeneity was high (I-squared 61.3%).

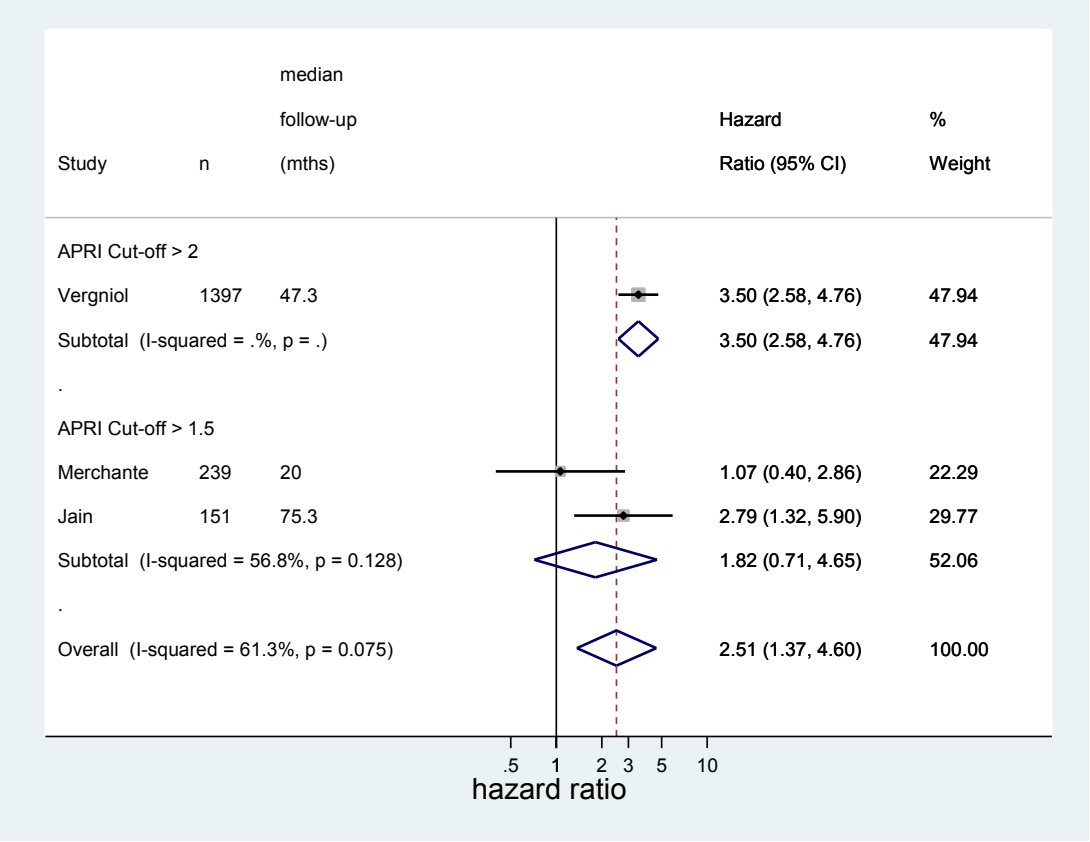


Figure 3-3 Meta-analysis of APRI to predict liver related death in Hepatitis C patients

3.5.3 Random effects meta-analysis of AST:ALT in patients with liver disease to predict overall death:

Meta-analysis of two (Bhala et al., 2011, Changchien et al., 2008) studies was possible using adjusted HR data, although the AST:ALT ratio cut-offs were different between the two studies (>1 and >2). The summary HR was not statistically significant at 1.93 (0.92-4.07), and there was a large proportion of between-study heterogeneity (I-squared) 49.1%. Results are illustrated in Figure 3-4.

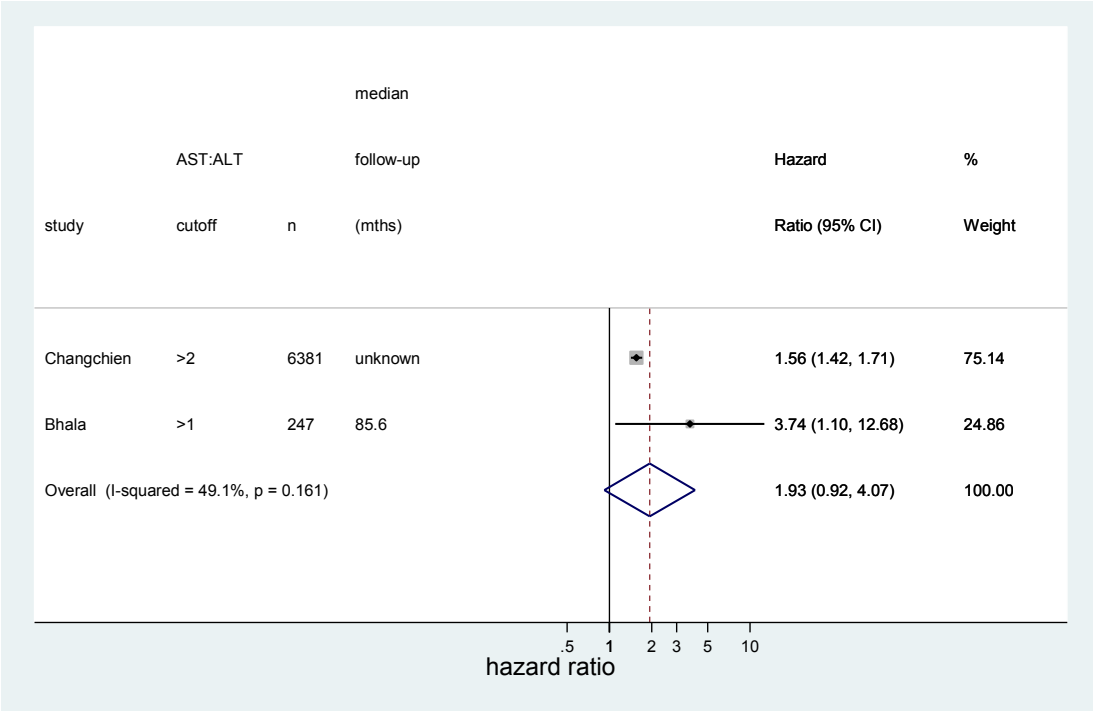


Figure 3-4 Meta-analysis of AST:ALT to predict overall death in patients with liver disease

3.5.4 Random effects meta-analysis of Fib-4 in patients with viral liver disease to predict overall death:

Three studies were included (Vergniol et al., 2011, Jain et al., 2012, Merchante et al., 2012) in the meta-analysis and all used the same cut off of 3.25. The unadjusted summary HR was 3.697 (1.977-6.914) (I-squared 73.5%) and the results are illustrated in Figure 3-5.

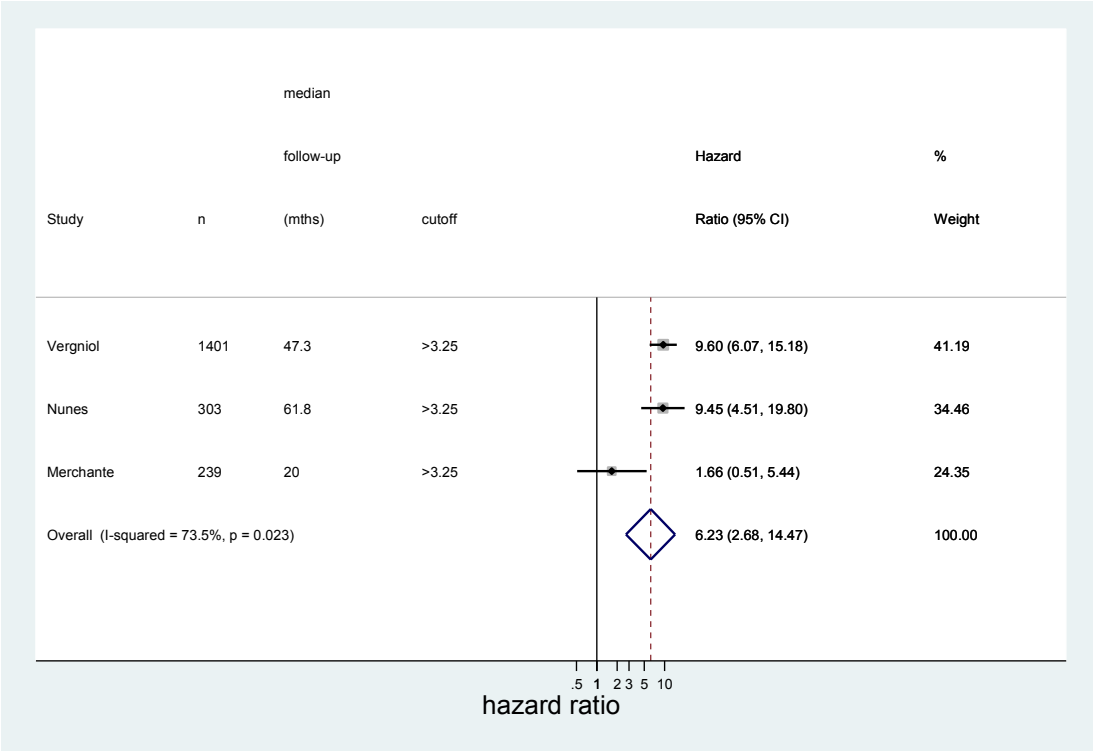


Figure 3-5 Meta-analysis of Fib-4 to predict overall death in viral liver disease patients

3.5.5 Random effects meta-analysis of Fib-4 in patients with viral liver disease to predict liver-related death:

Three studies were included (Merchante et al., 2012, Nunes et al., 2010a, Vergniol et al., 2011) in the meta-analysis all using the same cut-off of 3.25. The unadjusted summary HR was 6.23 (2.68-14.47) (I-squared 60.5%), and the results are illustrated in Figure 3-6.

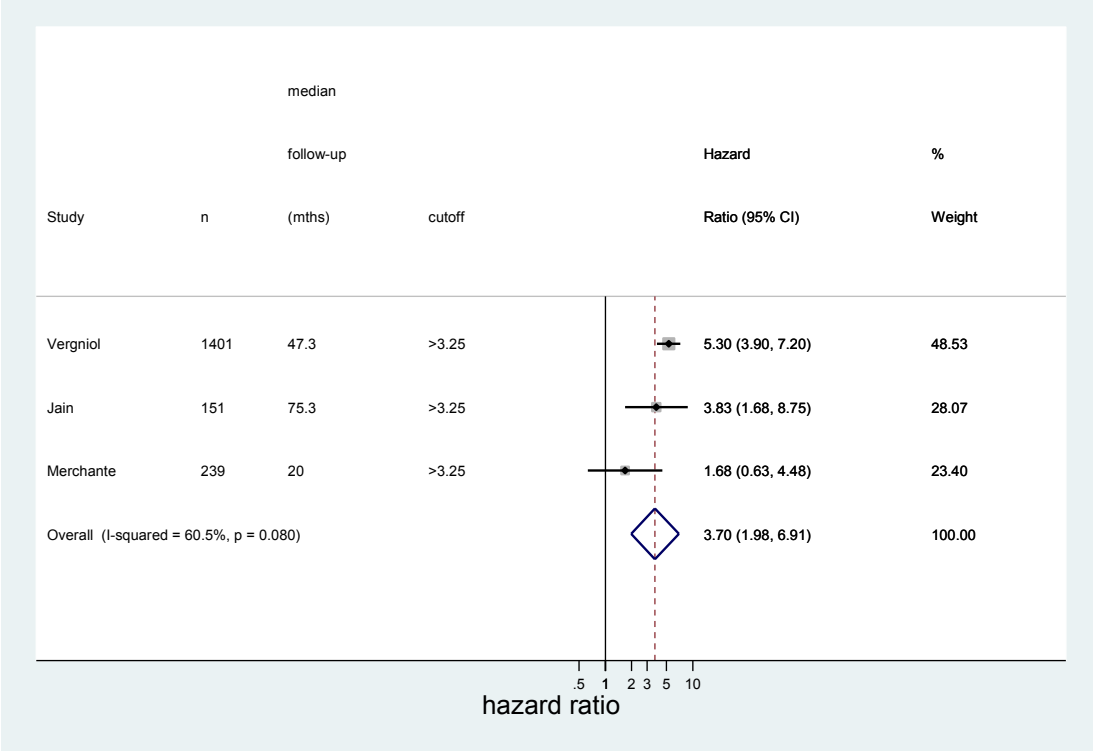


Figure 3-6 Meta-analysis of Fib-4 to predict liver related death in viral liver disease patients

Analysis of APRI (total patients, n=3726) and Fib-4 (n=3734) data included three papers for each, although both included data from 239 patients by Merchante et al (Merchante et al., 2012) for whom the overall and liver-related mortality figures included liver transplantation as part of their end-point. To specifically focus on mortality as a true end-point, we repeated the random-effect analysis with the Merchante et al data excluded, and the positive HRs for overall and liver-related death remained significant.

3.6 Discussion

Whilst there has been extensive study of non-invasive biomarkers to identify the presence and extent of liver fibrosis few studies have focussed on their potential prognostic value for subsequent non-liver and liver-related clinical events. Use of such biomarkers to predict an individual's risk of clinical outcomes would enable health care specialists to prioritise interventions for those at greatest risk.

This study represents a comprehensive analysis of the published literature. Our systematic review identified 31 studies that had used non-invasive biomarkers to predict clinical events in patients with liver disease. Although the studies displayed a large degree of heterogeneity with regards to biomarker use, methodology and study population our systematic review reinforced the prognostic value of certain biomarkers. At present the most compelling data relate to, APRI and Fib-4 which have the most robust evidence as prognostic factors for death in patients with viral hepatitis.

Simple scoring systems (APRI, AST:ALT, Fib-4) and specialist non-invasive tests (ELF, transient elastography) were consistently associated with the development of HCC and hepatic decompensation over a mean follow-up period of 45.7 months. In patients with viral hepatitis, our random-effects meta-analysis models demonstrated that both APRI (cut off of >1.5-2.0) and Fib-4 (cut-off>3.25) were associated with increased overall and liver-related mortality, over an average of 45.3 and 43.0 months respectively. This finding has potentially important implications for the management of such patients.

In the case of AST:ALT ratio, although there were two studies with significant HR for death, there is caution about its interpretation due to the high inter-study heterogeneity. In particular, the latter made the resultant confidence interval cross 1, therefore making the resultant HR of uncertain prognostic value.

Akin to the findings of the Poynard meta-analysis (Poynard et al., 2011), we show that biomarkers hold potential prognostic information that may be useful to clinicians wishing to understand how the prognosis of clinical outcomes is affected by an abnormal result. AUROC analysis aids discrimination of patients, but is not optimal in predicting future risk or stratifying individuals into risk categories. Our larger review and analysis has improved on previous studies by providing HR information showing the size of prognostic effect that elevations in Fib-4 and APRI have on clinical outcomes.

3.6.1 Limitations of this study:

Many of the studies reviewed primarily focused on the diagnosis of fibrosis rather than the prognosis of clinical outcomes and hence data presented in them is often not complete or could not be extracted for this purpose. The search strategy in itself is a limitation of the study. Some papers may have been missed by the incorrect spelling or non-standard method of describing terms searched for. Since our search was performed, there have also been 2 major studies that have been released by Kim at al(Kim et al., 2013) and Angulo et al(Angulo et al., 2013) that may have re-enforced the meta-analysis. They have also shown that in NAFLD patients, advanced fibrosis, as determined by non-invasive fibrosis marker

panels, is a significant predictor of mortality, mainly from cardiovascular causes, independent of other known factors

The major limitation of this study is the relatively small number of studies and the marked variation in their design, which precluded their inclusion in some of the meta-analyses. With the exception of APRI, Fib-4 and AST:ALT, many of the other biomarkers studied could not be meta-analysed as either the data were not provided to obtain HRs or the clinical/methodological heterogeneity was considered too severe to justify pooling. The latter was due to several discrepancies between the study designs and objectives, including: a wide range of clinical outcome measures (HCC, decompensation, mortality, varices, encephalopathy), disease types (viral, autoimmune, cancer), study design (prospective vs. retrospective), continuous values vs. variable biomarker cut-offs, and importantly a paucity of published HR. Nevertheless, this study does provide novel information on the prognostic value of pooled studies providing a platform for further investigation. Moreover, formal assessment of publication bias (e.g. funnel plot asymmetry) was not possible due to the small number of studies in each meta-analysis, which remains a concern as such bias is a recognised issue in prognosis studies. (Riley et al., 2013). Finally, most of the analysable data were from patients with viral liver disease and as such generalisation to other causes of liver disease should be undertaken with caution.

3.6.2 Future research:

Future multi-centre collaborative studies, based on similar study populations using validated and standardised measures such as REMARK guideline (Altman et al., 2012) or STROBE

(http://www.strobe-statement.org) to improve interpretability are likely to provide clearer insights as multiple small unrelated studies have the potential to add further to the uncertainty (Riley et al., 2013). Prospective validation of the thresholds for each biomarker will be key to generating robust conclusions. However, the greatest utility, and challenge, of such biomarkers is likely to be in primary care, where the absence of liver biopsy has limited the validation of biomarkers in liver fibrosis. However, assessing their prognostic value in this setting could greatly aid clinicians with prioritising their management, and at the same time aid health care commissioners in estimating the future clinical burden of liver disease.

CHAPTER 4 NON-INVASIVE MARKERS OF LIVER FIBROSIS. A PROSPECTIVE STUDY TO INVESTIGATE NEW MARKERS.

4.1 Introduction

Cirrhosis is the common endpoint of an aberrant repair process that results from uncontrolled, chronic inflammation and liver injury (Brenner et al., 2010, Friedman, 2000, Iredale et al., 1998, Iredale, 2003) The aetiology of chronic liver disease is varied but if left untreated all can lead to scarring of the liver (cirrhosis) and its associated complications such as ascites, portal hypertension and hepatocellular carcinoma.

The ability to assess the severity of fibrosis is important clinically to predict prognosis, and to plan and assess response to therapy. For example patients with severe fibrosis should be screened for complications of cirrhosis whereas those with no or mild fibrosis need less intensive follow up.

Traditionally the main way of assessing liver fibrosis has been the liver biopsy, which is an invasive test that carries with it a small but definite complication rate. Patients who undergo liver biopsy are put at risk of bleeding and pain post procedure with a small mortality rate. Liver biopsy is also open to inter-reviewer differences in scoring and interpretation and sampling errors because such a small piece of liver tissue is assessed.

These issues have prompted the search for non-invasive methods of assessing liver fibrosis including simple blood tests that reflect liver damage and dysfunction and as the pathogenesis of liver injury became better understood the use of groups of molecules that directly reflect fibrinolysis and fibrinogenesis as well as those that reflect underlying inflammation and immune activation. Recent interest has also focussed on novel imaging techniques including transient elastography, which uses ultrasound to measure fibrosis non-invasively.

The purpose of this study was to assess the accuracy of a panel of immune and fibrosis associated gene products in the diagnosis and assessment of liver fibrosis compared to established non-invasive markers of fibrosis and liver histology.

4.1.1 Background

4.1.1.1 Molecular mechanisms of liver fibrogenesis

There are many different causes of liver injury including infections, toxic injury, autoimmune diseases and ischemic damage. Many of these will resolve spontaneously allowing liver repair and regeneration to occur without scarring or permanent damage. However if the injury persists or is repetitive chronic liver disease results characterised by chronic inflammation (hepatitis) and scarring (fibrosis) develops which can progress over time to cirrhosis.

The response to repetitive injury in the liver is wound healing, which is characterised by chronic inflammation and fibroblast activation leading to the increased production and deposition of extracellular matrix. In the wound healing response, increased ECM is a normal and appropriate response to injury but excessive accumulation of ECM due to persistent or repetitive injury, myofibroblast activation and a failure of resolution and remodelling leads to disruption of normal tissue architecture and consequent loss of function. The constituents of the matrix laid down during liver fibrogensis are complex and include basement membrane and interstitial collagens, proteoglycans, and matrix glycoproteins such as fibronectin and laminin. The deposited matrix is remodelled by stromal cells and

macrophages, which secrete both matrix degrading enzymes (MMPs) and their inhibitors, (TIMPs). MMPs are zinc-dependent endopeptidases that degrade a range of matrix proteins and also modulate the function of cytokines and growth factors that are critical for regeneration, repair and wound healing. Excessive or aberrant wound healing leads to cirrhosis.

4.1.1.2 Markers of liver fibrosis

As discussed in the introduction, several indirect markers of fibrosis have been studied over the last few years. These include a mixture of commonly measured biochemical markers of liver injury and function and clinical features of liver disease. Direct markers are those that are released into blood as a direct consequence of fibrogenesis and include ECM components, matrix modulating enzymes and their inhibitors and markers of immune activation and inflammation that drives fibrosis.

In this project we compared different types of biomarkers, some of which are commercially available, others of which are experimental with indirect markers against the gold standard of histological assessment of fibrosis. Some biomarkers are specifically marketed to assess fibrosis (ELF and Fibrotest), but we also chose a panel of novel markers that reflect other potential roles during chronic inflammation and fibrogenesis.

The rationale behind the choice of each of these markers is explained below. Where studies have looked at levels of a particular marker in the past, this is described. Once all the markers were measured, statistical analysis was used to assess the ability and accuracy of individual or combinations of serum markers to predict fibrosis, with the aim of creating a new non-invasive composite biomarker.

4.1.2 Currently Available Commercially marketed markers of fibrosis

4.1.2.1 Fibromax

Fibromax consists of three tests combined. These are Fibrotest measuring hepatic fibrosis stage, SteatoTest measuring steatosis and NashTest assessing necroinflammation along a Kleiner NAS score.

The panel consists of:

Alpha 2 macroglobulin

- Haptoglobin
- Apolipoprotein A1
- Total Bilirubin
- AST
- ALT
- Gamma GT
- Fasting glucose
- Total Cholesterol
- Triglyceride (TG)

Fibrotest has been validated in patients with HCV, HBV, ALD and NAFLD in a meta-analysis with the AUROC for the diagnosis of bridging fibrosis (F2/F3/F4 vs. F0/F1) with AUROC data shown in table 4-1 (Halfon et al., 2008).

Condition	AUROC for F0-1 vs. F2-4
HCV	**0.84 (0.82-0.87)**
HBV	**0.81 (0.78-0.83)**
ALD	**0.87 (0.82-0.92)**
NAFLD	**0.84 (0.76-0.92)**

Table 4-1 Published AUROCs for Fibrotest

4.1.2.2 ELF

ELF consists of

- Hyaluronic acid
- Procollagen 3
- Tissue Inhibitor of Metalloproteinase 1

It has been shown to accurately predict moderate to severe fibrosis in several aetiologies of chronic liver disease.

For chronic HCV the pooled AUROC for prediction of Ishak 4-6 fibrosis was 0.85 (0.81-0.89) (Parkes et al., 2011).

In PBC, ELF to predict cirrhosis has an AUROC of 0.76 (0.63-0.89) or significant fibrosis with an AUROC of 0.75 (0.67-0.82).(Mayo et al., 2008)

In NAFLD, ELF can predict severe fibrosis, moderate fibrosis and no fibrosis with AUROC's of 0.90, 0.82 and 0.76 respectively.(Guha et al., 2008).

4.1.3 Imaging

Ultrasound allows for a rapid and non-invasive assessment of liver structure including the vasculature and biliary tree. It is possible for operators to give an assessment of liver disease based on the appearance of liver parenchyma. Excessive fat accumulation in hepatocytes results in an “echo-bright” signal and the architectural changes of cirrhosis give rise to a nodular or irregular liver edge. Recently transient elastography (Fibroscan TM) has been developed by EchoSens in France to provide a simple and non-invasive test for the presence of liver fibrosis. It transmits a low frequency vibration into the tissue inducing an elastic shear wave through the liver, the speed of which correlates with liver stiffness. The scan can be adversely affected by ascites, necro-inflammation(Kim et al., 2012a) or extrahepatic cholestasis(Millonig et al., 2008). MRI elastography has also been used, although mostly in a research setting. This is more expensive and requires more sophisticated interpretation but it has the advantage that when combined with spectroscopy it can also allow assessment of hepatic fat content(Huwart et al., 2006).

4.2 Rationale for markers assessed

Below we have set out what evidence there is for potential links between the markers we assessed and liver fibrosis.

4.2.1 Cytokines and markers of immune activation

4.2.1.1 CD40 ligand (CD154)

CD154 is a member of the TNF superfamily expressed on and secreted by activated T cells and endothelial cells. When it binds as a trivalent ligand to its receptor CD40 on target cells it provides a co-stimulatory signal for activation and differentiation of dendritic cells and B cells. In experimental animals, blocking CD40/CD40L co-stimulatory pathway prevents the development of allograft rejection in several models of organ allograft rejection. In humans, CD40 ligand is expressed by intrahepatic Kupffer cells and macrophages as well as T cells and it has been implicated in hepatocyte and cholangiocyte killing by activating CD40 dependent apoptosis in these cells during liver injury(Afford et al., 1999, Afford et al., 2001, Gaweco et al., 1999).

One study has found no increased in CD154 levels in the blood in chronic liver disease compared to normal controls (Mayo et al., 2006). Although CD154 messenger ribose nucleic acid (mRNA) in the liver correlated with the quantity of mRNA for secretory Ig.

4.2.1.2 TNFα

This cytokine has pro-inflammatory and immunoregulatory properties. It is primarily produced at site of inflammation by activated monocytes and macrophages. Elevated levels of TNF alpha are seen in acute and chronic liver disease and the main source in the liver is felt to be the Kupffer cells and infiltrating macrophages. It promotes stellate cell activation and increases MCP-1 production. It has mixed properties as mentioned above in that although it stimulates production of fironectin, it reduces the synthesis of types 1 and 3 collagen. Its main role in hepatic fibrogenesis is probably the activation of stellate cells(Reeves and Friedman, 2002). Levels in healthy controls are <4pg/ml which is elevated to >10pg/ml in alcoholics(Gonzalez-Quintela et al., 2008) or patients affected by HCV(Zylberberg et al., 1999). In patients with cirrhosis, elevated levels are seen compared to controls(Lin et al., 2007)

4.2.1.3 Adiponectin

Adiponectin is a hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism but which also acts as a potent immune modulating cytokine. It has antilipogenic and anti-inflammatory effects. Low levels of circulating adiponectin are associated with several manifestations of the metabolic syndrome, including insulin resistance and type 2 diabetes mellitus as well as NAFLD and NASH(Havel, 2004). Hypoadipponectinaemia has also been demonstrated to be a feature of NASH compared to simple steatosis allowing for differentiation.(Hui et al., 2004)

Circulating adiponectin is increased in liver cirrhosis independent of the aetiology (Kaser et al., 2005) and in subjects with more advanced chronic liver disease, suggesting it is an indicator of severity of chronic liver disease. Adiponectin levels correlate positively with surrogate markers of hepatic fibrosis (transient elastography, fasting serum bile acids and hyaluronate) as demonstrated by Balmer et al in 2010. The respective R values were 0.45, 0.51 and 0.52. (Balmer et al., 2010)

4.2.1.4 Insulin

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) is a method for assessing beta-cell function and insulin resistance (IR) from basal (fasting) glucose and insulin. The relationship between glucose and insulin in the basal state reflects the balance between hepatic glucose output and insulin secretion. Type 2 diabetes is more prevalent among patients with chronic HCV compared with those with other liver diseases and the general population. IR plays a primary role in the development of type 2 diabetes mellitus. A 2003 study by Hui et al(Hui et al., 2003) showed that Hepatitis C virus–infected subjects with stage 0 or 1 hepatic fibrosis had higher levels of insulin and HOMA-IR, and that increased HOMA-IR is a predictor of the stage of fibrosis and the rate of fibrosis progression. A study of HCV patients from Egypt illustrates the correlation between HOMA-IR and fibrosis stage(Mohamed et al., 2011). Another study found that HOMA-IR remained an independent predictor of fibrosis stage even after the exclusion of subjects with cirrhosis, which is known to cause IR and impaired insulin clearance.(Kruszynska et al., 1993)

In cirrhotics, the more severe the disease, the lower the HOMA-IR that is detected (see below)(Yagmur et al., 2006) and this is lower than seen in healthy controls.

4.2.1.5 Leptin

Adipose tissue secretes substances called adipocytokines such as adiponectin, leptin, resistin, plasminogen activator inhibitor 1 (PAI-1), and TNF-α. Activated stellate cells have also been shown to express leptin, which acts as a paracrine modulator of fibrinogenesis(Potter et al., 1998). Recently it has been proposed that obesity is a low grade inflammatory state contributing to insulin resistance and type 2 diabetes.(Greenberg and Obin, 2006) Hypertrophied adipocytes in obesity release chemokines such as TNF-A, Il6 and nitric oxide, which recruit macrophages. This in turn leads to adipocytokine dysregulation.

High serum leptin levels are seen in cirrhotic patients(Testa et al., 2000) and hepatic cirrhosis is six times more prevalent in obese individuals than in the general population(Ratziu et al., 2000). The relationship between serum leptin concentrations and the severity of liver fibrosis is unclear. Some studies suggest that despite high serum leptin concentrations in NAFLD patients, there is no relationship between leptin and the severity of hepatic fibrosis(Angulo et al., 2004), however Lemoine et al. measured serum leptin levels in 74 patients with biopsy-proven disease (57 NASH, 17 simple steatosis) and found significantly higher levels in the NAFLD group (14.3 ±11.1 ng/ml) compared with the control (5.8 ±6.6 ng/ml) .(Lemoine M, 2009) In another study, serum leptin levels in the liver were not significantly different between patients with NASH and those with simple steatosis.(Chalasani et al., 2003)

In HCV patients, a positive correlation is seen not only with fibrosis stage but also body mass index (BMI).(Piche et al., 2004) Levels in control samples (6.4 ±4.1 ng/ml) were lower than patients. Males had lower levels compared to females (4.1 ±3.2 vs. 8.7 ±3.6 ng/ml).

These studies suggest that leptin plays important roles in liver diseases by attenuating hepatic steatosis, exacerbating liver fibrosis, and possibly promoting HCC growth.

4.2.1.6 Resistin

Resistin is a polypeptide of 12.5kDa. It reduces insulin sensitivity in adipocytes, skeletal muscle and hepatocytes by supressing insulin stimulated glucose uptake. It therefore aids in maintaining blood glucose levels during fasting by activating hepatic gluconeogenesis. Higher serum resistin levels are found in obese individuals and diabetic patients. It is also expressed in leukocytes and tissue macrophages. Observing increased levels of resistin in the presence of Il-1, Il-6 ad TNF-a, supports this.

In viral hepatitis patients, significantly higher levels of resistin are seen comparing patients with F4-6 vs. F0-3.(Tsochatzis et al., 2008). Levels of resistin varied according to aetiology. Hepatitis C and B patients had values of 7.1 ng/ml ± 2.5, whereas NASH levels were lower at 5.7 ±2.8. No controls were used.

In another study from a few years earlier, the same link was seen (Yagmur et al., 2006). Here a control population of healthy donors yielded a value of 4ug/l. In addition, a link was seen with increasing resistin levels correlating with increase in model for end-stage liver disease (MELD) score.

4.2.1.7 IFN-γ

IFN-γ inhibits the in vitro activation of stellate cells (Baroni et al., 1996) and their synthesis of extra cellular matrix(Reeves and Friedman, 2002). In murine models IFN-γ deficient mice have a more pronounced fibrotic response to carbon tetrachloride damage, which was reduced by administration of IFN-γ(Shi et al., 1997). Things may not be as straightforward however, as a study performed on liver biopsy specimens from patients with HCV infection, showed that increased IFN-γ expression was associated with portal inflammation and fibrosis stage(Napoli et al., 1996). This reflects the dichotomous effects of IFNg on immune activation and hepatitis and fibrosis per se.

Serum levels are elevated in patients with primary biliary cirrhosis were 44pg/ml compared to 19pg/ml when measured in non-cirrhotics (Fracchia et al., 2000). Interestingly levels are not elevated in chronic carriers of HBV, which may reflect the lack of inflammation (Tangkijvanich et al., 2000).

4.2.2 Chemokines

4.2.2.1 RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted)

RANTES (or CCL5) is a cytokine that attracts several subsets of activated lymphocytes and monocytes that express its G-protein coupled receptors C-C motif receptor 1 (CCR1), CCR3 and CCR5. CCR5 is expressed by hepatic stellate cells suggesting that the cells are a target as well as a source for CCL5 in the liver (Schwabe et al., 2003). In a murine CCL5 knockout model, decreased hepatic fibrosis was observed with reduced stellate cell activation and

immune cell infiltration in 2 models of liver injury: CCl4 toxicity and MCD (methionine choline-deficient) diet induced steatohepatitis. (Berres et al., 2010) This study also showed a link with fibrosis stage in HCV/NASH and fibrosis indicating that CCL5 expression is associated with fibrotic liver disease.

4.2.2.2 Serum Interferon-gamma-inducible protein 10 (IP-10)

Serum interferon-gamma-inducible protein 10 (IP-10) is also called C-X-C motif chemokine (CXCL)10. It is a chemokine that plays a central role in liver inflammation by recruiting effector lymphocytes to the liver(Shields et al., 1999, Curbishley et al., 2005, Oo and Adams, 2010) and is elevated in flares of Hepatitis B and C as well as in PBC. CXCL10 binds to the chemokine receptor CXCR3 as do two related chemokines CXCL9, and CXCL11. These chemokines were measured in healthy controls, patients with liver fibrosis and cirrhosis of various disease aetiologies to investigate their role in disease progression.(Tacke F, 2010)CXCL9 and CXCL10 but not CXCL11 levels were positively associated with the severity of liver fibrosis and CXCL10 was elevated across all stages of cirrhosis. In addition, CXCR3 chemokines were associated with hepatic synthetic capacity and the development of clinical complications of cirrhosis. IP-10 has been found to predict response to current Hepatitis C treatment (Ribavirin and Pegylated Interferon).(Reiberger T, 2008). CXCL10 levels also correlate with the presence of necroinflammation which is characterised by CXCR3+ lymphocyte infiltrates(Zeremski et al., 2008) .In mice CXCL10 deficiency is associated with attenuated fibrosis in the CCl4 model. It was also noted that HSCs express CXCR3, respond to CXCL10 and secrete CXCL10 when stimulated with IFN-G(Hintermann et al., 2010).

In a study looking at fibrosis development 1 year after liver transplant for HCV, a positive correlation between CXCL10 levels and fibrosis stage was observed Levels ≤140pg/ml significantly predicted the absence of F2 fibrosis and a level of ≤220pg/ml did the same for F3(Berres et al., 2011). Elevated levels of CXCL10 correlated with ALT levels as might be expected given the above findings in several inflammatory liver diseases(Nagayama et al., 2001).

CXCL10 in murine models

In the murine model of CCl4 fibrosis CXCL10 deficiency was associated with reduced fibrosis compared with wild type mice. It was also noted that NK cells numbers were raised and that HSCs express CXCR3, respond to CXCL10 and secrete CXCL10 when stimulated with IFN-γ (Hintermann et al., 2010).

4.2.2.3 CXCL11/ Interferon-inducible T cell alpha chemoattractant (I-TAC)

This cytokine, also known by the name I-TAC is expressed by leukocytes and detected in the liver. It interacts with CXCR3 and is chemotactic for activated T cells(Cole et al., 1998).

In patients with mixed cryoglobulinaemia and chronic HCV, significantly higher levels of CXCL11 were seen in the blood (Antonelli et al., 2011) and even higher levels were seen in those patients with an active vasculitis. In the Berres study looking at fibrosis development 1 year after liver transplant for HCV, a positive correlation between CXCL10 and to a lesser extent CXCL11 levels and fibrosis stage was observed (Berres et al., 2011).

4.2.2.4 MCP-1

MCP-1 (also known as CCL2) is a potent chemotactic factor for monocytes and macrophages. Its sources include both intrahepatic and peripheral mononuclear cells. CCL2 secretion is up-regulated during chronic hepatitis and correlates with the number of cells infiltrating the portal tract(Marra F and M, 1998). It is elevated in alcoholic liver disease and reflects severity of hepatic inflammation (Fisher et al., 1999). One study looked at MCP-1 polymorphisms and noted that HCV patients who are genetically predisposed to produce greater amounts of MCP-1 protein are more prone to hepatic inflammation and fibrogenesis.(Mühlbauer et al., 2003). Measurement of this may direct more aggressive therapy toward those patients with an increased risk of disease progression. In patients with acute liver failure, elevated levels of MCP-1 are observed (see below) over controls and patients with chronic liver failure(Roth et al., 2009).

Levels of MCP-1 vary between studies, probably reflecting different assays used. In a study of HCV patients, controls had levels of MCP-1 of 209.56-±26.33 pg/ml and patients with cirrhosis had levels of 192.75-±59.52 pg/ml whereas those with HCC had elevated levels of 302.67 ±44.52 pg/ml. These differences were not however significant(Tachibana et al., 2007).

4.2.2.5 Interleukin 1B

Il-1B is a pro-inflammatory cytokine generated in response to inflammatory activation. It stimulates B cell proliferation, growth of fibroblasts and the induction of adhesion

molecules, cytokines and chemokines by a broad range of cell types. In one study, IL-1B levels were not significantly increased in cirrhotic patients compared with controls.(Napoli J, 1994) and other studies have reported lower Il-1B levels compared to controls. Spanakis et al describe mean levels of Il-1B of 22pg/ml in controls, which was reduced in patients with HCV(Spanakis et al., 2002).

4.2.2.6 Il-6

Il-6 plays an important role in regulating cell growth and differentiation as well as the immune response. It binds to the Il-6 receptor which is a protein complex consisting of IL-6 receptor (Glycoprotein 80) subunit binding to Gylcoprotein 130 (GP 130). GP 130 is a transmembrane protein and is important in signal transduction after the cytokine engages. Once this occurs, it interacts with Janus kinases to create an intracellular signal. This initiated intracellular signalling is strictly dependent on the function of GP130 (Ishihara and Hirano, 2002).

Il-6 has been shown to induce fibroblast proliferation and collagen production as well as synthesis of TIMP. Elevated Il-6 levels are seen after partial hepatectomy suggesting a role in hepatocyte proliferation and liver regeneration(Matsunami et al., 1992). In Il-6 deficient mice treated with CCl4, fibrotic changes were less evident than amongst controls and TGFB expression was also reduced(Natsume et al., 1999, Kovalovich et al., 2000).

Levels in human subjects are significantly elevated in patients with cirrhosis compared to controls(Migita et al., 2006) . HCC was associated with more frequently elevated levels of Il-6 and also in patients with cirrhosis compared to HBV carriers(Tangkijvanich et al., 2000).

4.2.2.7 Il-10

Il-10 is an anti-inflammatory cytokine primarily produced by monocytes, which plays an important role in regulating and terminating inflammatory and immune responses. Il-10 deficient mice are more prone to hepatic fibrosis in the presence of helminthic infection(Mentink-Kane et al., 2011) and CCL4 (Louis et al., 1998). Il-10 has also been implicated in the mechanism of action of stem cell therapy in mice (Suh et al., 2012).

A study from 2000 in Gastroenterology showed that in humans, administration of Il-10 reduces hepatic inflammation and liver fibrosis in the presence of HCV(Nelson et al., 2000). When the final study was reported, elevated levels of HCV were seen in patients despite lower levels of fibrosis. This was due to changes in immunologic viral surveillance(Nelson et al., 2003). In a study of patients with cirrhosis(Lin et al., 2007), elevated levels of Il-10 were observed over controls.

4.2.2.8 Il-12 p70

Il-12 is produced by stimulated macrophages and B cells and promotes the development of Th1 polarised immune responses characterised by interferon gamma production and cell-mediated immunity. IFN feeds back on T and NK calls to stimulate more Il-12 thereby amplifying the inflammatory response to infections. It is a heterodimer of two chains p35 (light chain) and p40 (heavy chain) resulting in the active 70kDa (p70) form of Il-12. Il-12 deficient mice infected with trypanosomes show reduced transaminases and liver inflammation(Barkhuizen et al., 2008). Mice deficient in Il-10 show increased expression of IFNg and Il-12 in response to Toxoplasma infection (Gazzinelli et al., 1996). And Il-12 is down regulated in IFN gamma knockouts (Tsuji et al., 1999). In humans, elevated levels have been

documented (see below) in patients with alcoholic hepatitis and cirrhosis(Tung et al., 2010). Controls had mean levels of 39.3 pg/ml ±8.3, with cirrhotics having mean levels of 110.5 pg/ml ±41.6. Il-12 has also been implicated in the pathophysiology of PBC(Hirschfield et al., 2009). The binding of Il-12 to its receptor is proposed to modulate autoimmune responses by stimulating interferon gamma production.

4.2.2.9 Il-13

Il-13 is a TH2 cytokine known to induce fibrosis through the regulation of TGFβ production and activation(Fichtner-Feigl et al., 2007, Lee et al., 2001). Lee et al showed using a transgenic (Tg) mouse that over-expresses IL-13 in the lung, that IL-13 is a potent inducer of MMP-9 and TGF-β1 expression. The activation of TGF-β1 is mediated by a MMP-9-dependent mechanism. They also showed that when TGF-β activity is neutralized, collagen deposition in the lungs of the IL-13 Tg mice is substantially decreased. Thus indicating a direct functional link between IL-13 and TGF-β. In murine schistosomiasis howeverIl-13 exert its profibrogenic role independently of TGFβ.(Kaviratne et al., 2004) A study by Weng et al in 2009 showed that both TGFβ and Il-13 are associated with fibrosing CLD, but that their impact differs depending on the aetiology of the damage(Weng et al., 2009). They also showed that elevated levels of Il-13 are observed in patients with HCV compared to controls (72.87pg/ml ±26.83 vs. 45.41pg/ml ±3.73)

4.2.2.10 Il-17

IL-17 is the signature cytokine of a family of proinflammatory cytokines that play an important role in the immune response to extracellular pathogens. Il-17 is also implicated in

autoimmune and destructive inflammatory diseases. In murine models, increased levels of Il-17 and its receptor are seen in response to liver injury. Il-17 then activates inflammatory and liver resident cells and facilitates production of Il-6, Il-1 and TNF alpha. It has been observed to directly induce production of collagen in HSC by activating the Stat 3 signalling pathway(Meng et al., 2012).

In humans, Il-17 is mainly produced by CD4+ TH17 cells, but can be produced by CD8+ T cells, monocytes, neutrophils and eosinophils. Its receptor is widely found on epithelial and stromal cells(Oo and Sakaguchi, 2013). Il-17 increases expression and secretion of certain chemokines (CXCL1, 6 and 8) as well as growth factors granulocyte colony-stimulating factor (GCSF) and granulocyte macrophage colony-stimulating factor (GMCSF), and Il-6 to mobilise, recruit and activate neutrophils. In a study looking at patients with alcoholic related liver disease, elevated levels of plasma Il-17 have been observed compared to patients with ALD (median 55.9pg/ml) and healthy subjects (0 pg/ml) (Lemmers et al., 2009).

4.2.3 Markers of tissue injury

4.2.3.1 Cytokeratin-18 (CK-18)

In vitro experiments have shown increased cellular release of cytokeratin 18 fragments into the extracellular space as a consequence of caspase activation during the intermediate stage of apoptosis. CK18 is the major intermediate filament protein in the liver and one of the most prominent substrates of caspase during hepatocyte apoptosis(Linder et al., 2004) CK-18 has been studied for its ability to distinguish simple fatty liver from NASH and several

studies show that CK-18 fragments independently predict the presence of NASH and correlate with the magnitude of hepatocyte apoptosis and disease severity.(Feldstein et al., 2009, Wieckowska et al., 2006) CK-18 has also been found to elevated in cholestatic liver disease, viral hepatitis, hepatocellular carcinoma and alcoholic hepatitis.(Yilmaz, 2009)

M65 is associated with overall cell death and M30 is associated with apoptotic cell death(Kramer et al., 2004). The ratio between them is an indication of the proportion of apoptosis compared to total cell death. Interpreting the ratio can be difficult, but a low level (ratio of <5%) of cleaved CK18 (M30) in the presence of high levels of CK18 indicates a high component of necrotic cell death whereas a ratio of >20% may indicate apoptosis. It is this that may aid in the diagnosis of NASH vs. NAFLD. Studies are on going into its clinical efficacy though.

Recently it has been shown by Joka at al that the M65 assay had a better correlation with fibrosis and steatosis(Joka et al., 2012).

CK18 has been used to predict recovery from acute liver failure(Volkmann et al., 2008). Elevated levels of M30 were associated with spontaneous recovery whereas elevated levels of M65 were associated with a worse outcome suggesting that caspase activation and apoptosis is associated with survival and thus Implicating a caspase independent cell death pathways in irreversible forms of liver failure.

4.2.4 Endothelial adhesion receptors

4.2.4.1 Vascular Adhesion Protein 1 (VAP-1)

VAP-1 is a homodimeric transmembrane sialoglycoprotein and amine-oxidase enzyme, which is constitutively expressed by hepatic sinusoidal endothelial cells and high endothelial venules. It is also expressed as a soluble protein in the serum. It is released from cytoplasmic stores where it is important in mediating the interaction and binding of lymphocytes to the endothelium. VAP-1 expression increased with chronic inflammation in the vessels of tonsils, gut, skin and synovium.

Soluble VAP-1 is generated by the cleavage of membrane bound protein from the surface of endothelial cells and adipocytes. Circulating VAP-1 accounts for most of the amine oxidase activity in both humans and mice. Its role is uncertain, but it has been shown to be elevated in certain liver diseases, particularly in patients with alcoholic liver disease (Jeff's 16/17).(Weston and Adams, 2011)

The concentrations of VAP-1 measured by Enzyme-linked immunosorbent assay (ELISA) in healthy individuals were found to be between 49-138 ng/mL(Kurkijärvi et al., 1998). The difference of sVAP-1 concentrations between the liver patient group and normal controls was statistically highly significant (*p,* 0.0001). Moreover, when comparisons were made according to the nature of the liver disease, differences within the liver disease group became apparent. The highest values of circulating soluble VAP-1 was found in patients with active cirrhosis due to ALD but not in those with acute liver damage from paracetamol poisoning. Thus, increased circulating levels of soluble VAP-1 are associated with chronic hepatic inflammation rather than acute liver injury.

VAP-1 activity rather than total VAP-1 level is also elevated in certain disease conditions such as diabetic atheroscelrosis(Magyar and Meszaros, 2003). However, when the vessels are homogenised and tissue activity is assessed, there was an inverse correlation with elevated serum levels and decreased tissue levels (Nunes et al., 2010b). In liver tissue work in our lab has shown that serum VAP-1 activity correlates well with total serum VAP-1(Trivedi et al., 2013). PSC was also noted to have higher levels of tissue VAP-1 activity compared to other diseases such as PBC or AIH.

4.2.5 Profibrotic growth factors

4.2.5.1 Fibroblast growth factor basic (FGFb)

FGFb is present in basement membranes and sub-endothelial matrix of blood vessels. During wound healing and tumour formation, heparan sulphate degrading enzymes activate FGFb leading to angiogenesis(Rusnati and Presta, 1996). Elevated levels of basic FGF are seen in chronic liver disuse(Jin-no et al., 1997). Very high levels were seen in hepatocellular carcinoma (HCC) patients (see below), however its use as a marker for HCC is limited due to lack of specificity and also that in healthy patients, co-existent acute illness significantly raises FGF(Hsu et al., 1997).

4.2.5.2 Vascular endothelial growth factor (VEGF)

This growth factor promotes angiogenesis and is produced by hepatocytes in response to tissue injury when it is associated with proliferation of endothelial cells and elevated levels have been observed after partial hepatectomy (Taniguchi et al., 2001).

In humans, elevated levels of VEGF are observed in acute hepatitis and levels correlate with rises in transaminases (Akiyoshi et al., 1998), but VEGF levels are reduced in cirrhotics with mean levels (see below) of 63 ng/l in cirrhotics, 360 in controls and 1123 in acute hepatitis(Assy et al., 1999). Within cirrhotics however, there was no difference in levels seen depending on Childs score.

4.2.5.3 Serpin E1

Serpin E1, also known as PAI-1 is a serine protease inhibitor that inhibits tissue plasminogen activator (tPA) and urokinase. These are the principal activators of plasminognen and hence fibrinolysis. It is an acute phase protein that can be induced during inflammation(Bergheim et al., 2006). PAI-1 is known to be induced in models of hepatic fibrosis and is directly produced by stellate calls when activated in vitro(Leyland et al., 1996). In a model of bile duct ligation in PAI-1 knockout mice, significant protection against accumulation of ECM was seen. Alongside the increased expression of activated stellate cells and collagen synthesis seen with the injury, there was increased activity of tPA. This suggests that PAI-1 plays a causal role in fibrosis during cholestasis. This is largely due to activation of tPA-induced HGF, which is a known antifibrotic agent. Elevated matrix metallopeptidase 9 (MMP9) were seen, along with increased collagen degradation(Bergheim et al., 2006, Wang et al., 2007).

Different models of damage have however shown contradictory results. In a CCL4 model, fibrosis was enhanced in PAI-$1^{-/-}$ mice after chronic CCl4 administration. Indeed, all indices of liver damage were elevated in PAI-$1^{-/-}$ mice compared with wild-type mice. This enhanced liver damage correlated with impaired hepatocyte proliferation. These data suggest that PAI-1 may play multiple roles in chronic liver diseases, both protective and damaging, either

mediated by its influence on inflammation and fibrosis or helping maintain hepatocyte division after an injury. (von Montfort et al., 2010)

Levels of PAI-1 in humans are noted to be lower in patients with obesity (10.63 ±4.82) compared to controls (14.26 ±11.4)(Espino et al., 2011)in a small group of Hispanic patients and that there was no difference in plasma levels amongst obese patients with liver fibrosis compared to those without fibrosis.

4.2.5.4 Hepatocyte growth factor (HGF)

HGF plays an important role in hepatic regeneration and is increased in liver injury. It is expressed in non-parenchymal liver cells such as endothelial and Kupffer cells and in other organs including the lungs and kidneys(Maher, 1993). It is mainly eliminated via the liver. Serum HGF is elevated in most types of liver disease and correlates with inflammatory activity in chronic hepatitis.in cirrhotic patients those with Childs C disease have significantly higher levels than patients who are Childs A or (Shiota et al., 1995).

HGF has also been shown in two HCV cohorts to be correlated with fibrosis stage.(Marin-Serrano et al., 2010, Imbert-Bismut et al., 2001). These findings contrast with TGFB1 lower levels of which are seen as fibrosis stage increases.

4.2.6 Other markers of immune activation

4.2.6.1 Monoclonal immunoglobulin serum free light chains (FLC)

Serum FLCs are comprised of kappa (κ) and lambda (λ) light chains are elevated in many B-cell dyscrasias, including multiple myeloma, AL amyloidosis and patients with monoclonal gammopathy of undetermined significance, typically producing an abnormal κ/λ FLC ratio.

Abnormal FLC ratios have also been described in patients with chronic lymphocytic leukaemia and non-Hodgkin's lymphoma(Martin et al., 2007). Increased concentrations of κ and λ FLCs can result from reduced clearance by the kidneys or increased polyclonal production by plasma B cells. Polyclonal elevations of FLCs have been reported in a number of autoimmune diseases including systemic lupus erythematosus (Aggarwal et al., 2011), Sjögren's syndrome(Gottenberg et al., 2007) as well as in HIV and lymphoma(Landgren et al., 2010). Abnormal B-cell activation is also observed in autoimmune liver diseases, shown by the emergence of autoantibodies and high levels of immunoglobulins detected in many chronic liver diseases(Zeman and Hirschfield, 2010).

In addition, aberrant B cell function is observed in chronic HCV infection where persistence of the virus leads to chronic stimulation of B cells resulting in the production of autoantibodies. In some patients, this can lead to malignant transformation of B cells resulting in lymphoma development. Monitoring FLC levels in such individuals may prove clinically useful if abnormalities in FLC expression define individuals pre-disposed to lymphoma formation.

FLCs are normally metabolised and filtered by the kidneys, however, immunoglobulins and FLCs are excessively produced in patients with B cell dyscrasias, and many patients suffer kidney dysfunction, leading to significant impairment in the removal of these FLCs. Kidney dysfunction is also associated with liver disease. For example, the key pathophysiological hallmark of hepatorenal syndrome, which occurs in patients with advanced chronic liver disease, is the vasoconstriction of the renal circulation in these patients(Arroyo and Fernandez, 2011).

Furthermore, acute renal failure is a common complicating factor in patients with liver cirrhosis. These abnormalities in kidney function are likely to impact on the concentrations of FLCs in liver disease. A preliminary study has shown that FLCs are elevated in patients with chronic liver disease(Assi et al., 2010) hence the need to collect samples from a larger population to determine the potential diagnostic and prognostic utility of FLC measurements in liver disease.

4.3 Methods

4.3.1 Study Population

All patients referred for an outpatient liver biopsy between September 20111 and 2012 were identified using the NHS pre-admission clinics booking list. Patients were sent an information sheet through the post and then consented at the clinic appointment. This appointment was usually 2-3 days prior to the biopsy having taken place and allowed a nursing checklist to be taken along with blood tests and observations. If patients consented to take part in the study, they underwent a physical examination and Fibroscan.

Local ethical approval was obtained

Appendix 1 contains copies of all of the relevant study materials such as consent forms, patient information sheets, General Practitioner letters and ethical approval letters.

4.3.2 Data collection

Demographics, anthropometric measurements and observations were recorded at the clinic visit along with TE measurements. For TE, the probe type, number of successful acquisitions, success rate and interquartile range (IQR) were recorded. If the patient had had a valid Fibroscan within 2 months, this was not repeated.

4.3.3 Fibroscan

In our unit, all operators underwent a certified training session (4 hour) with an Echosens consultant prior to using the M-probe and/or XL-probe in the clinical setting. Prior to

certification, the operators performed 3 TE examinations on volunteers with the Echosens consultant in attendance to determine if the application of the TE probe and interpretation of the LSM were correct.

TE was performed using either the M-probe (3.5 Hz frequency) or XL-probe (2.5 Hz frequency) with the Fibroscan® 502 machine (Echosens, France). The manufacturer recommends that the XL probe should be used in patients with a skin-capsular distance >2.5 cm (measured by sonographic imaging). Due to the time constraints in liver clinic, operators were advised to use the XL-probe in patients with a measured BMI > 30 Kg/m^2 (Myers et al., 2012) the Fibroscan® 502 Touch (Echosens, France), which has a built-in automated indicator that recommends the probe best suited to the patient's morphology. In accordance with manufacturer's guidance, all TE examinations are performed in our clinics with the patient lying in the dorsal decubitus position with the right arm extended. The tip of the transducer probe (covered with coupling gel) is placed on the skin in an intercostal space overlying the right lobe of the liver. A time-motion ultrasound image allows the operator to locate a portion of liver at least 6-cm thick and free of large vascular/bony structures. The median value of successful acquisitions (target = 10) is deemed to be representative of the liver stiffness, represented as LSM. If no value was obtained following 10 acquisitions LSM failure was documented. LSM was only classified as 'valid' if all three of the manufacturer's criteria(Castéra et al., 2010a) were met: 1) ≥10 successful acquisitions; 2) success rate was ≥60%; and 3) IQR/M ratio <30%. If any of these three criteria were not met the LSM was classified as 'invalid.'

4.3.4 Liver Biopsy Assessment

In our centre, liver biopsies are routinely reported using the appropriate disease-specific liver fibrosis staging (i.e. Ishak for hepatitis C; Kleiner for non-alcoholic fatty liver disease). For purposes of this study, however, each biopsy was re-assessed independently by two liver pathologists (NM & RB or NM & SH) without knowledge of LSM results or other clinical data. In cases of disagreement, a consensus was reached by a joint review. To take account of the diverse aetiologies of liver disease, liver fibrosis was staged using a modified version of the Ishak scoring system(Ishak et al., 1995), as previously described (Rosenberg et al., 2004). Significant fibrosis was defined as a modified Ishak score > 2 and advanced fibrosis as a modified Ishak score of 5 or 6. The length of biopsy specimens and the number of portal tracts sampled were recorded as measures of biopsy quality.

4.3.5 Blood tests

Routine clinically indicated blood tests including full blood count, clotting screen and a biochemical profile were taken once consent had been obtained and sent to the hospital laboratory for processing and analysis.

Blood for the Fibromax testing were packed unprocessed in a biohazard labelled box provided by the company and posted the same day.

The research blood vials were taken to the University of Birmingham (institute of Biomedical Research) and processed in the good clinical laboratory practice (GCLP) lab by lab technicians. Serum was obtained after a 10 min 2700RCF spin and frozen to -80 degrees Celsius immediately. Plasma and white cells were obtained using a lymphoprep gradient to separate the cells from rom the plasma. The spin speed was the same as that for the serum,

but with no centrifuge braking. Cells were pipetted off and stored in foetal calf serum and dimethyl sulphoxide (DMSO). Suspended cells were then transferred to a Mr Frosty freezing container to allow gradual reduction to -80 degrees Celsius over a 24 hours period. Cells were then transferred to a liquid nitrogen container and stored in the vapour phase for future use as required.

4.3.5.1 Enzyme-linked immunosorbent assay (ELISA) testing done in laboratory at University of Birmingham

Serum ELISAs were performed according to manufacturers guidelines (R&D, Mercodia and Peviva). Peviva produce the CK18 (M30 and M65) assays. Merdodia produce the Insulin assay and R&D manufactured the multiplex kits that all the other markers were analysed for. Serum VAP-1 was sent to two labs with whom we have long-standing collaborations: Biotie is a Finnish biotech company who have established VAP-1 assays; the Jalkanen lab at Biocity in Turku has established assays of VAP-1 and its SSAO enzyme activity(Kurkijärvi et al., 1998)

4.3.6 Collagen Proportionate Area (CPA)

Sections of each biopsy were stained with Van Gieson using an autostainer to reduce variability. The slides were then loaded into a Leica SCN-400 whole slide scanner. After whole section digital image capture (Figure 4-1), CPA was measured with Image J software. CPA measurement included steps to eliminate artefacts and structural collagen in large portal tracts and blood vessel walls. Unfilled spaces such as vascular cavities were not included in the measurements(Calvaruso et al., 2009). The slides had been scanned at 20x so any degree of magnification was possible up to this. Slides were zoomed into at 4x

magnification giving a scale of 100um/cm Closer zooming gave better images but required much more time input. Slides at lower magnifications were felt to not provide enough detail.

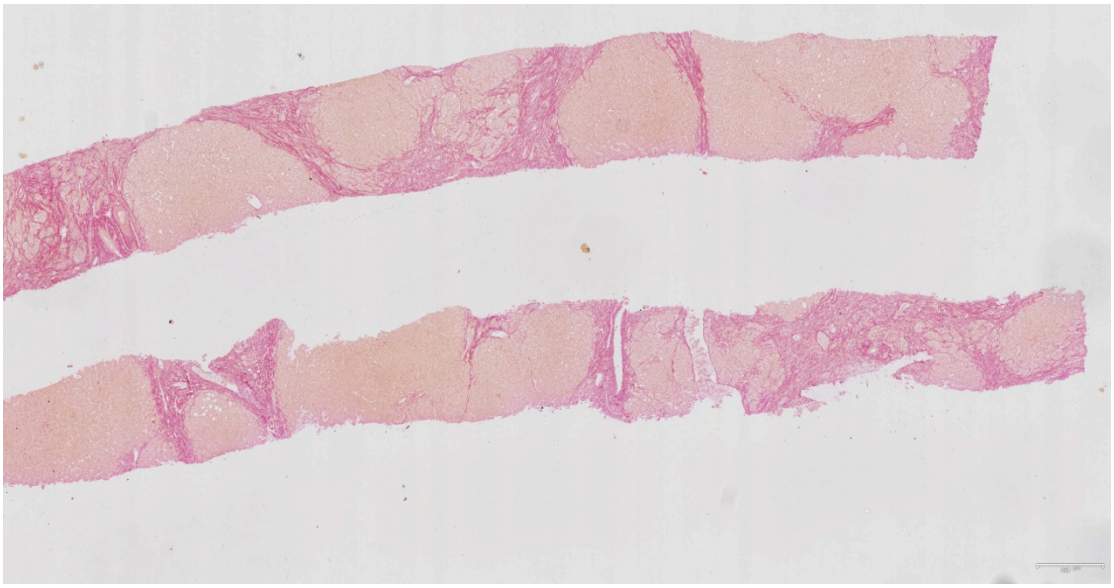


Figure 4-1 Van Gieson stained liver biopsy slide

A very high definition monitor (2560x1440) was used for this process to ensure the slides were split as few times as possible. Once the area of slide containing biopsy was highlighted, the remaining area was cleared. The total number of pixels was then measured.

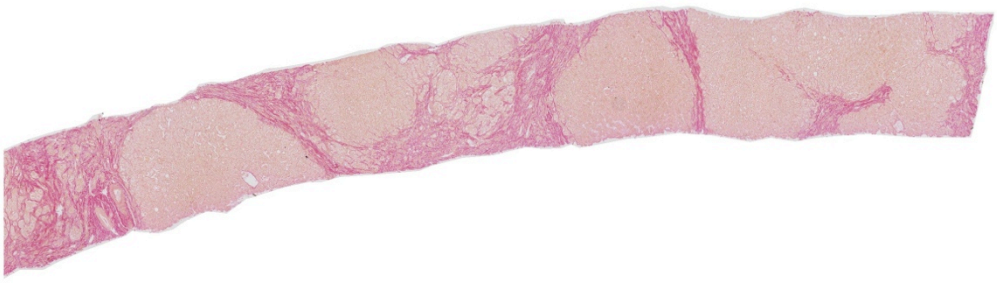


Figure 4-2 Stained slide after exterior cleared using Image J software

After clearing around the biopsy slide (Figure 4-2), the image was adjusted using the Image J tool (Hue, Saturation and Brightness) to ensure that the Van Gieson staining was highlighted only. Settings used were: Hue 166-255, Saturation 70-255 and Brightness 0-223. These settings were performed on a selection of biopsies across the Ishak stages to ensure the best all round result and were checked by two authors (MJA and SH). This process highlighted the fact that some biopsies from patients with advanced disease had lower than expected results and some normal biopsies appeared more diseased than anticipated, so each slide was treated using the same cut-offs as well as personalized ones to allow investigation of differing methodology. The eventual analysis of slides was done blinded to the Ishak stage to avoid any bias.

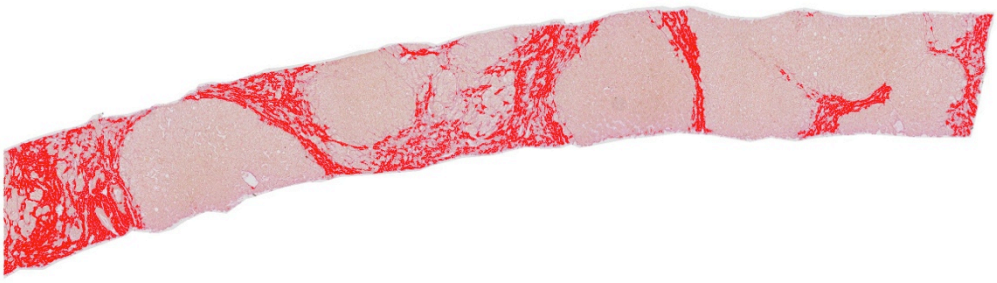


Figure 4-3 Slide after pixels highlighted using Image J software

Using Image J in the selected section, we highlighted pixels in red using the above settings. They were then selected using the tool and measured giving a percentage value.

Figure 4-4 Highlighted pixels now selected using Image J software

The whole of the biopsy would very often not be fitted on the screen so values were summed to create an overall result.

Once the Image J macro had been run, the resultant number of pixels was noted and divided by the earlier total to give a % area.

4.3.7 VAP-1 staining

A selection of slides from across the spectrum of Ishak stages were also stained for VAP-1 (Figure 4-5) in the lab and analysed using the same method. Samples were fixed in formalin and processed through to paraffin wax. Tissues were deparaffinised and rehydrated to water, and after a low temperature retrieval technique (ALTER) were immunostained on a Dako Autostainer using a Prestige rabbit-anti-human VAP-1 polyclonal antibody (Sigma, 1:200), a Vector ImmPRESS secondary reagent kit) and NovaRED as chromogen (both VectorLabs Inc.).

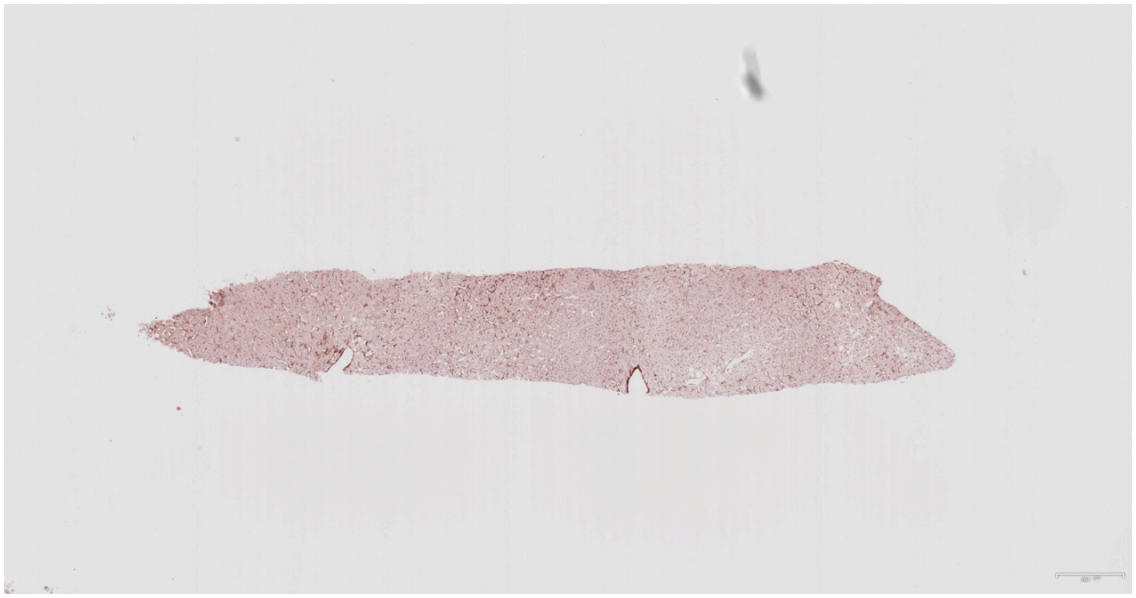


Figure 4-5 VAP-1 stained biopsy slide with VAP-1 staining appearing as darker purple areas

4.3.8 FLC and Cystatin C

Combylite is a new kit produced by a company called The Binding Site that measures the total concentrations of free kappa and free lambda light chains (FLC) in a single assay, and gives equivalent values to summated results from the Freelite kappa and lambda assays. The Freelite assay has been manufactured for over 12 years now and this measures the kappa and lambda concentrations separately. Freelite was run to determine if there may be an underlying monoclonal gammopathy in any of the Combylite high samples. Samples were analysed in the Binding Site laboratory in Birmingham where they were compared against healthy controls previously assessed in their lab.

4.3.9 Statistical analysis

Data were analysed using IBM SPSS 19, Graph pad Prism V5 and Microsoft excel with p values less than 0.05 deemed to be indicative of significance. Data were expressed as mean ± SEM or median ±SD unless stated otherwise. AUROC analysis was used to illustrate the accuracy of a certain marker to predict fibrosis stage.

4.4 Results

4.4.1 Demographics

127 patients were sent information sheets. Of these, 40 patients either declined to take part or did not turn up to the clinic slot. 87 patients were therefore consented to take part in the study. 2 patients then did not proceed to biopsy – one was taking aspirin and the other decided against biopsy. Final statistics were therefore performed on 85 patients. (43 male, 42 female) with a median age of 54. Median BMI was 30±7. Median waist to hip ratio was 0.989±0.17. Median time from being seen to biopsy was 2±4.7 days.

4.4.2 Fibroscan

The median time from Fibroscan to biopsy was 2±27 days. Fibroscan was performed in 77 patients with a median stiffness of 11kPa (IQR 13.1). 8 patients had a failed Fibroscan (no successful readings). There was a mean success rate of 87% ±2.8. The M probe was used in 31 cases and the XL in the other 46 cases. Median stiffness was 11.4 kPa ±12.4 with a median IQR value of 2.6±5.4. Median IQR as percentage of median was 22%±21.

A valid scan was generated if success rate was >60%, IQR/median <30% and ≥10 successful readings. 45 of the 77 (58%) were valid. Where valid only readings are used, the median success rate rose to 100% ±8.5 and median stiffness was 10.7kPa ±7.6 with a median IQR of 1.2 ±1.8

4.4.3 Blood and Histology results

The median ±SD value for the blood results are displayed in table 4-2.

Haematology		Chemistry		Liver	
Hb	13.8±1.3	Na	141±2.6	AST	38±49
WCC	6.5±2.1	K	4.2±0.35	ALT	53±82
Plt	201±66	Ur	5±1.4	ALP (old)	199 ±107
INR	1±0.07	Cr	70±16.8	ALP (new)	99±109
		Gluc	5.5±5	Alb	45±3.7
				Bili	9±43
				AFP	3±1.9
				GGT	53±108

Table 4-2 Blood results

Distribution of histological grade is displayed in figure 4-6.

Median Ishak stage was 2±1.79 and median biopsy length was 23.5cm±6.6.

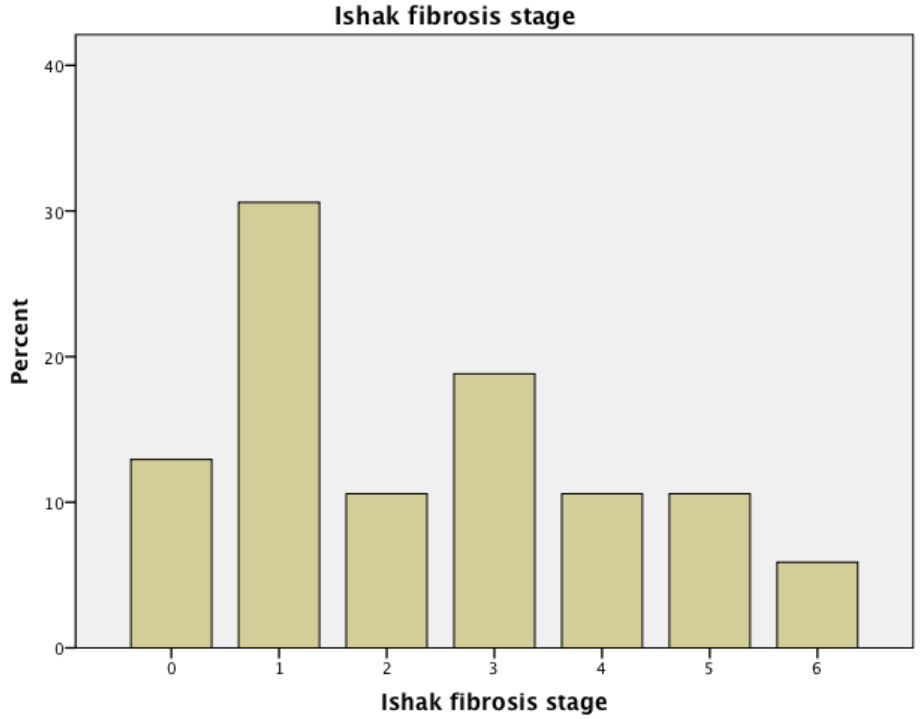


Figure 4-6 Distribution of histological grade

Over half of the patients who underwent biopsy had a diagnosis of fatty liver. Results are presented in table 4-3.

Diagnosis	N	Percent
Viral liver disease	15	17.7
Fatty liver	45	52.9
Autoimmune	13	15.3
PSC	1	1.2
Others	11	12.9

Table 4-3 Aetiology of liver disease

Correlations between Ishak stage and variables assessed are shown in tables 4-4 and 4-5 with table 4-5 being a subgroup analysis for the largest cohort with was fatty liver disease. Table 4-6 shows correlations for variable that have computed scores.

Variable	Spearman Correlation	Significance	N if <85
BMI	0.068	0.534	
Waist: Hip	0.172	0.116	
FS Stiffness valid	0.375	0.011	45
FS Stiffness all	0.240	0.035	77
Platelets	-0.355	0.001	
INR	0.140	0.200	
Alb	-0.221	0.042	
Bili	0.059	0.593	
CK18 M30	0.155	0.158	
VAP-1 Biotie	0.388	<0.0001	
VAP-1 Turku	0.350	0.001	
ELF	0.470	<0.0001	
Hyalauronic Acid	0.476	<0.0001	
P3NP	0.250	0.021	
TIMP1	0.204	0.061	
Combilyte	0.263	0.015	
VEGF	0.086	0.433	
TNF-A	0.299	0.005	
Il-10	0.150	0.171	
Il-6	0.079	0.472	
IFN-G	0.005	0.965	
FGFB	0.034	0.760	
MCP1	0.194	0.076	
RANTES	-0.216	0.047	
Leptin	0.009	0.936	
Adiponectin	0.128	0.244	
Resistin	0.127	0.248	
Serpin	-0.050	0.648	
HGF	0.125	0.256	
CXCL11	0.219	0.044	
CD40L	0.004	0.970	
CXCL10	0.388	<0.001	
Alpha2 Macroglobulin	0.531	<0.001	77
Haptoglobin	-0.213	0.069	74
Apolipoprotein A1	-0.158	0.170	77
Total Chol	-0.174	0.124	79
Triglyceride	-0.024	0.830	
VAP-1 & CXCL10	0.480	<0.0001	

Table 4-4 Correlations between Ishak stage and variables for all patients- VAP-1 and CXCL10 predicted results from regression analysis
No Il-17, Il-1B, Il12p70 or Il13 detected

Variable	Spearman Correlation	Significance	N if <45
BMI	0.006	0.967	
Waist: Hip	0.237	0.116	
FS Stiffness valid	0.425	0.043	23
FS Stiffness all	0.173	0.268	43
Platelets	-0.520	<0.0001	
INR	0.282	0.061	
Alb	-0.080	0.601	
Bili	0.279	0.063	
CK18 M30	0.297	0.047	
VAP-1 Biotie	0.557	<0.0001	
VAP-1 Turku	0.559	<0.0001	
ELF	0.591	<0.0001	
Hyalauronic Acid	0.591	<0.0001	
P3NP	0.265	0.078	
TIMP1	0.099	0.516	
Combilyte	0.337	0.024	
VEGF	-0.080	0.603	
TNF-A	0.236	0.118	
Il-10	0.196	0.198	
Il-6	0.121	0.427	
IFN-G	-0.055	0.719	
FGFB	0.040	0.792	
MCP1	0.050	0.746	
RANTES	-0.308	0.039	
Leptin	-0.053	0.729	
Adiponectin	0.001	0.994	
Resistin	0.007	0.964	
Serpin	-0.302	0.044	
HGF	0.038	0.806	
CXCL11	0.150	0.324	
CD40L	-0.087	0.572	
CXCL10	0.352	0.018	
Fibrotest	0.604	<0.0001	39
Alpha2 Macroglobulin	0.568	<0.0001	40
Haptoglobin	-0.450	0.004	39
Apolipoprotein A1	-0.043	0.794	40
Total Chol	-0.327	0.034	42
Triglyceride	-0.173	0.255	

Table 4-5 Sub Group analysis: Correlation between Ishak stage and test for Fatty liver patients (n=45) No Il-17, Il-1B, Il12p70 or Il13 detected

Variable	Spearman Correlation	Significance	N if <85
Forns	0.364	0.004	61
Fib4	0.411	<0.0001	82
APRI	0.299	0.006	82
AST:ALT	0.200	0.072	82
Hepascore	0.543	<0.0001	59
Fibrotest	0.510	<0.0001	75
ELF	0.470	<0.0001	
MELD	-0.134	0.220	

Table 4-6 Correlations between Ishak stage and computed scores for all patients

A Jonckeere-Terpstra analysis was performed which looks for trends across stages. Results are shown in table 4-7 and figures 4-7 through to 4-19.

Test	Jonckheere-Terpstra	Pairwise significances
Forns	0.004	
Fib-4	<0.0001	3-6 p=0.04
APRI	0.007	
AST:ALT	0.079	
Hepascore	<0.0001	
Fibrotest	<0.0001	0-4 p=0.044 1-6 p=0.026
ELF	<0.0001	1-5 p=0.002
Fibroscan	0.011	
CXCL10	<0.0001	1-5 p=0.040
VAP-1 Biotie	0.001	1-5 p=0.021
VAP-1 Turku	0.002	
Hyalauronic acid	<0.0001	1-5 p=0.004 1-6 p=0.023
P3NP	0.020	1-4 p=0.021
TIMP1	0.064	
Alpha 2 macroglobulin	<0.0001	0-3 p=0.034 0-4 p=0.004 0-6 p=0.023 1-6 p=0.028
Haptoglobin	0.054	
Apolipoprotein A1	0.177	

Table 4-7 Values of Tests and link to Fibrosis stage with non-parametric analysis

4.4.4 Histograms to illustrate median ±SD of markers with pairwise significance added where present

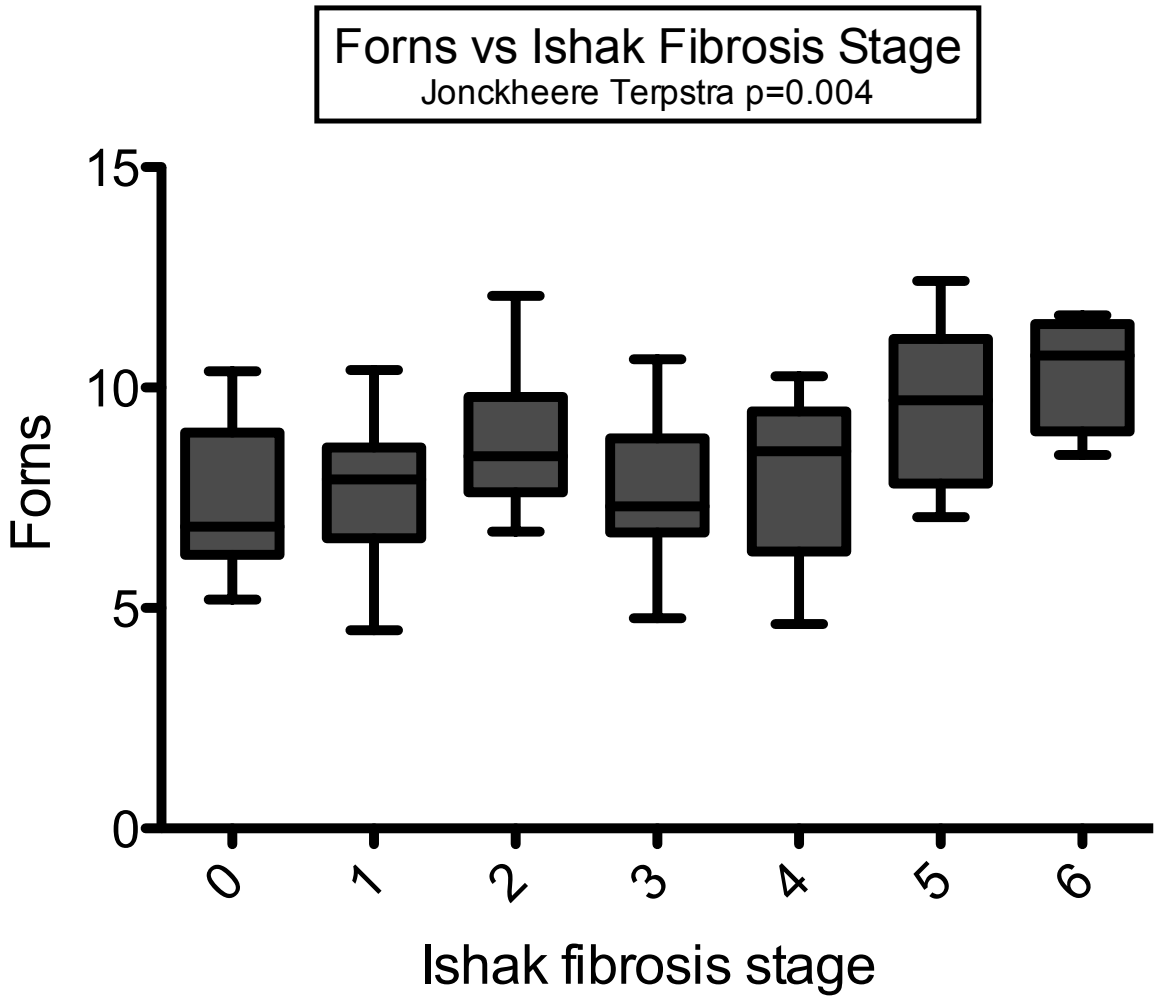


Figure 4-7 Forns vs. Ishak fibrosis stage showing distribution of Forns readings according to fibrosis stage

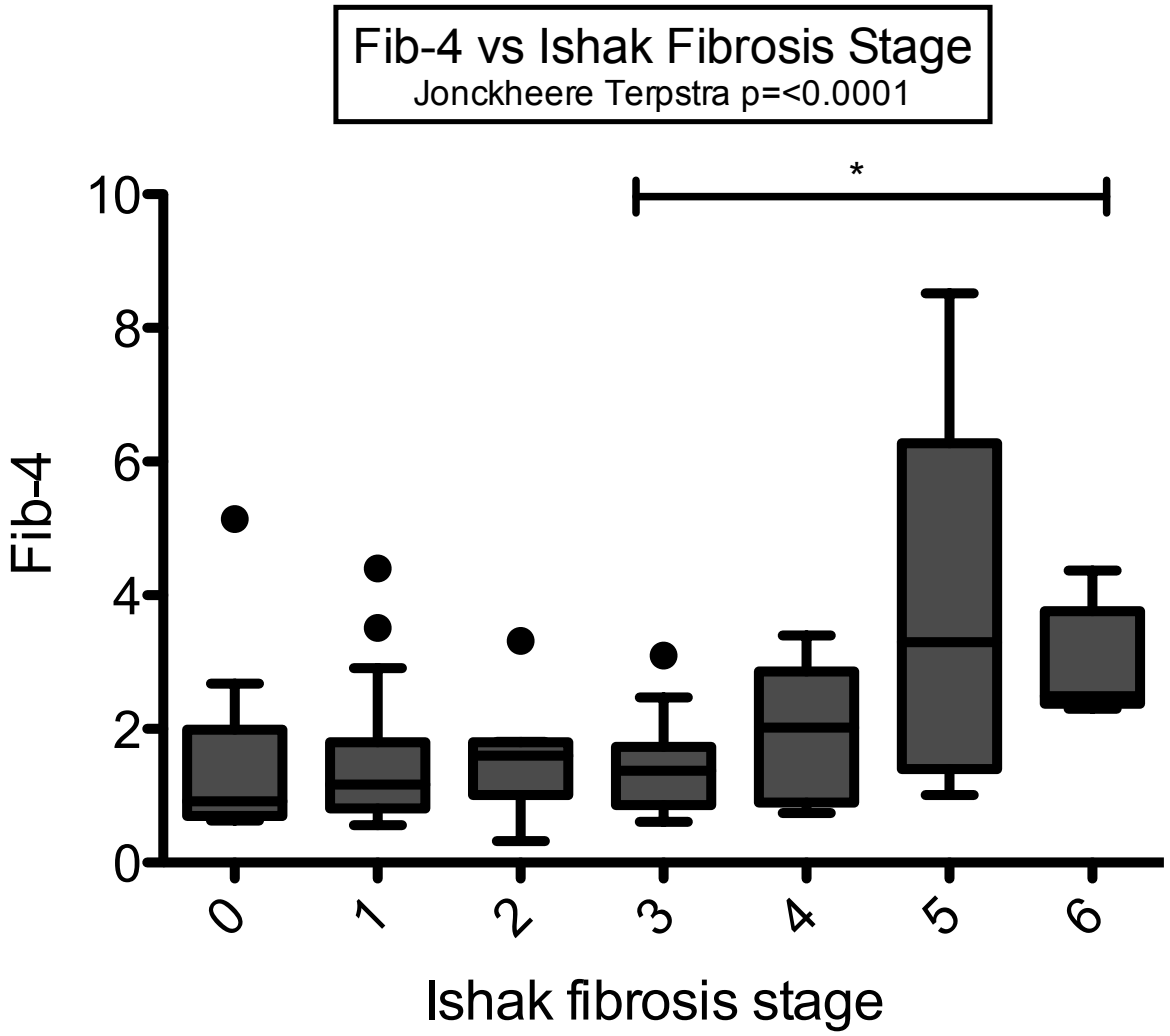


Figure 4-8 Fib-4 vs. Ishak fibrosis stage showing distribution of Fib-4 readings according to fibrosis stage. Pairwise significance seen between F3 and F6

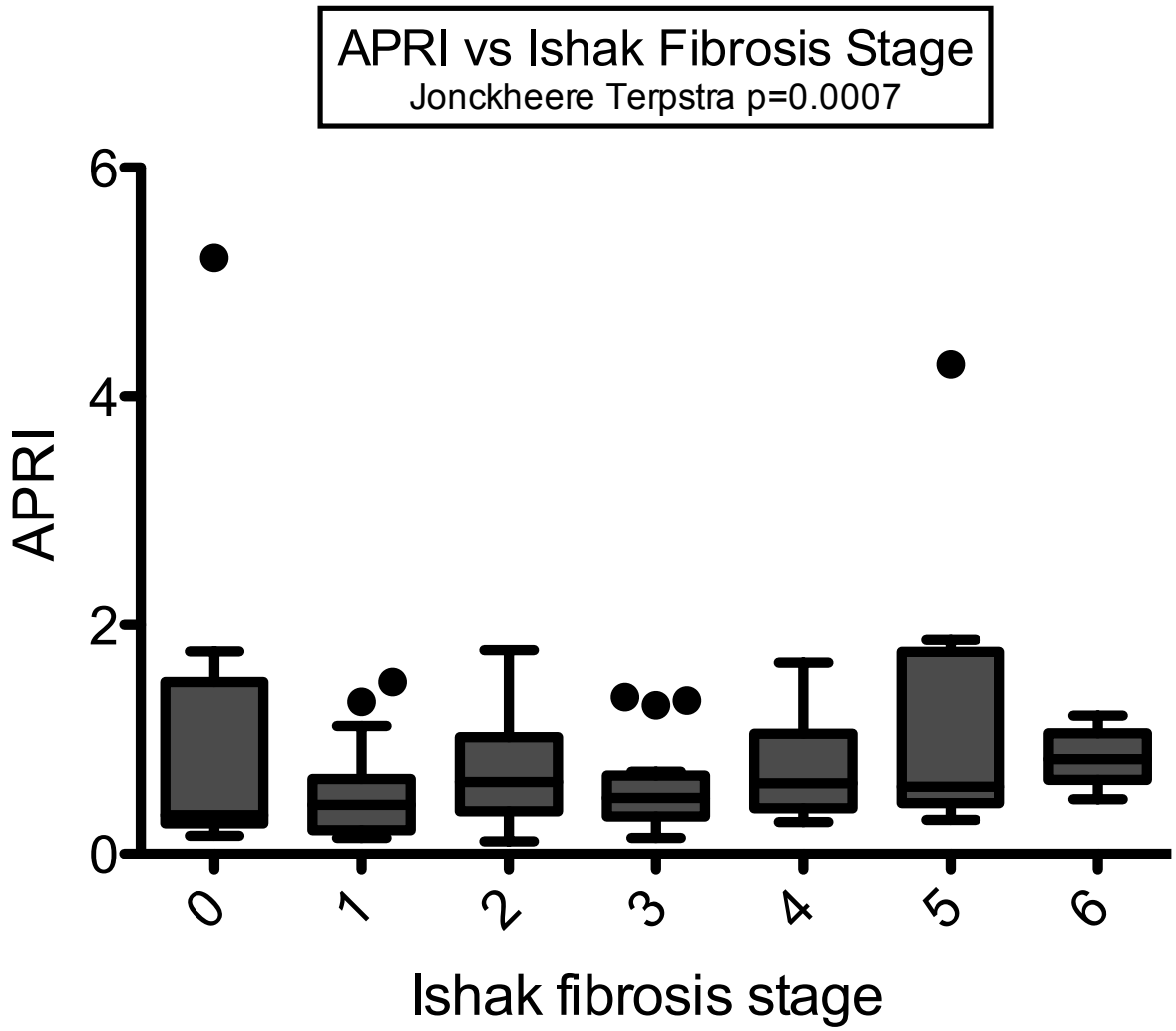


Figure 4-9 APRI vs. Ishak fibrosis stage showing distribution of APRI readings according to fibrosis stage

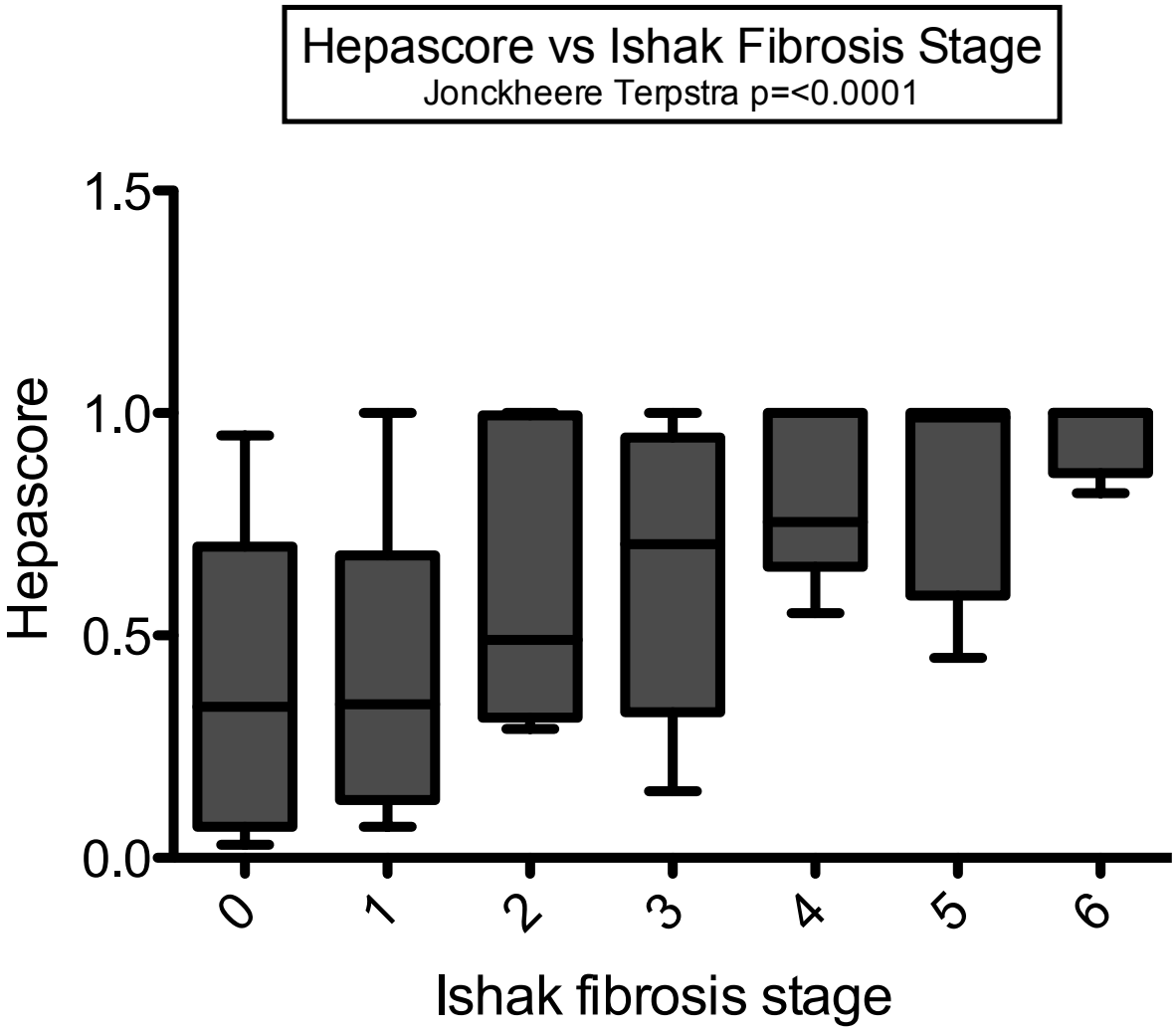


Figure 4-10 Hepascore vs. Ishak fibrosis stage showing distribution of Hepascore readings according to fibrosis stage

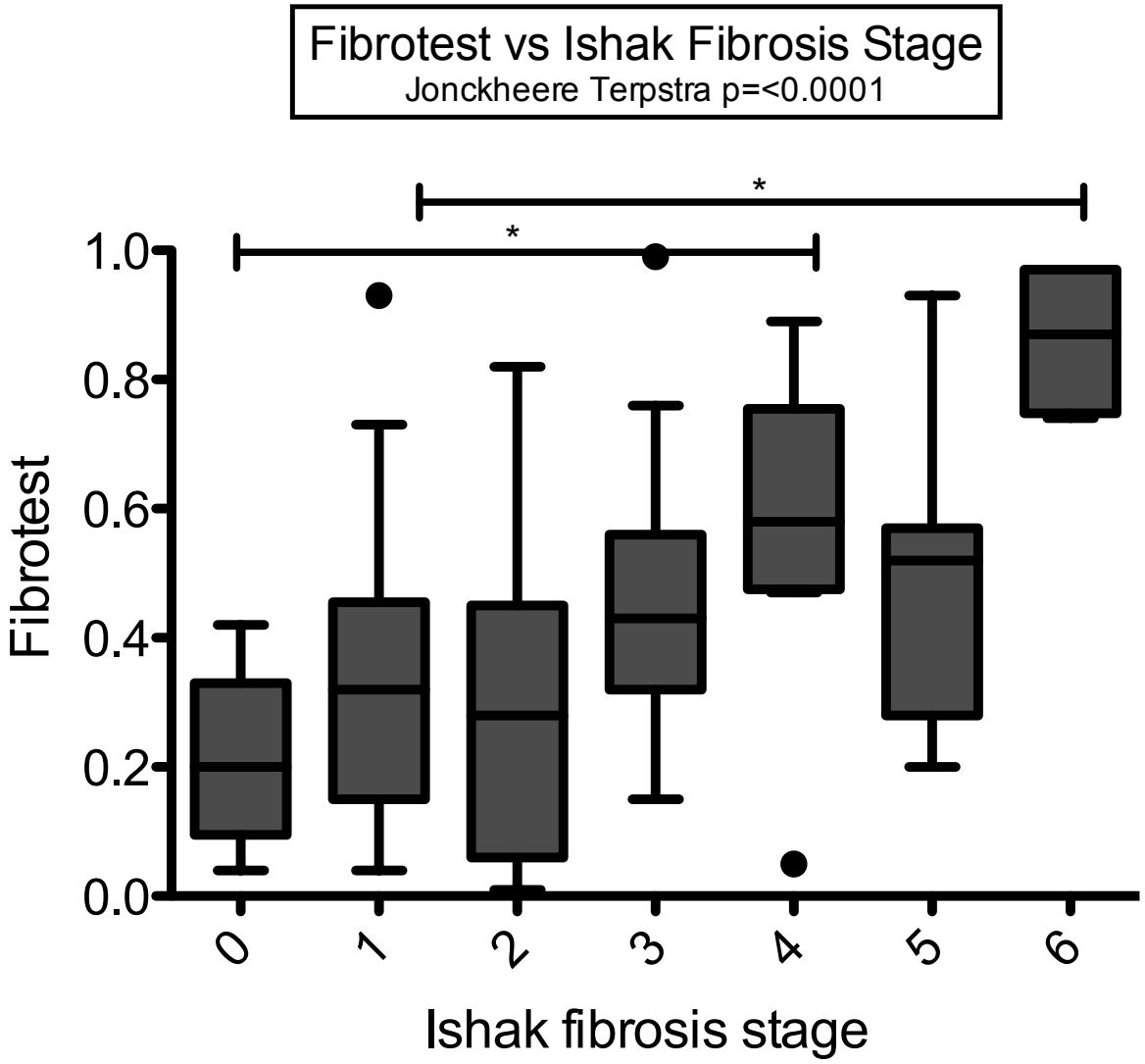


Figure 4-11 Fibrotest vs. Ishak fibrosis stage showing distribution of Fibrotest readings according to fibrosis stage. Pairwise significance seen between F0 and F4 as well as F1 and F6

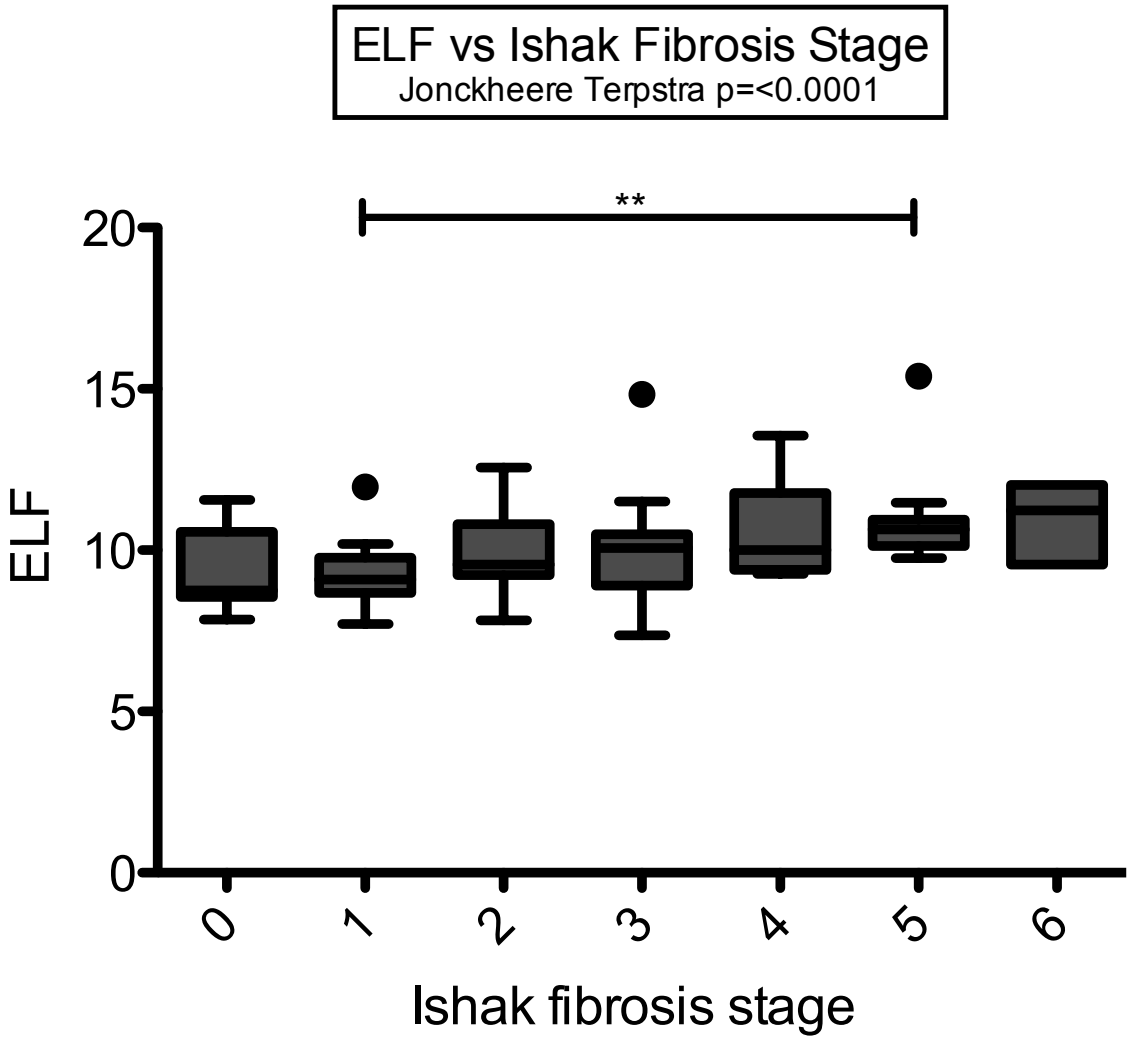


Figure 4-12 ELF readings vs. Ishak fibrosis stage showing distribution of ELF readings according to fibrosis stage. Pairwise significance seen between F1 and F5

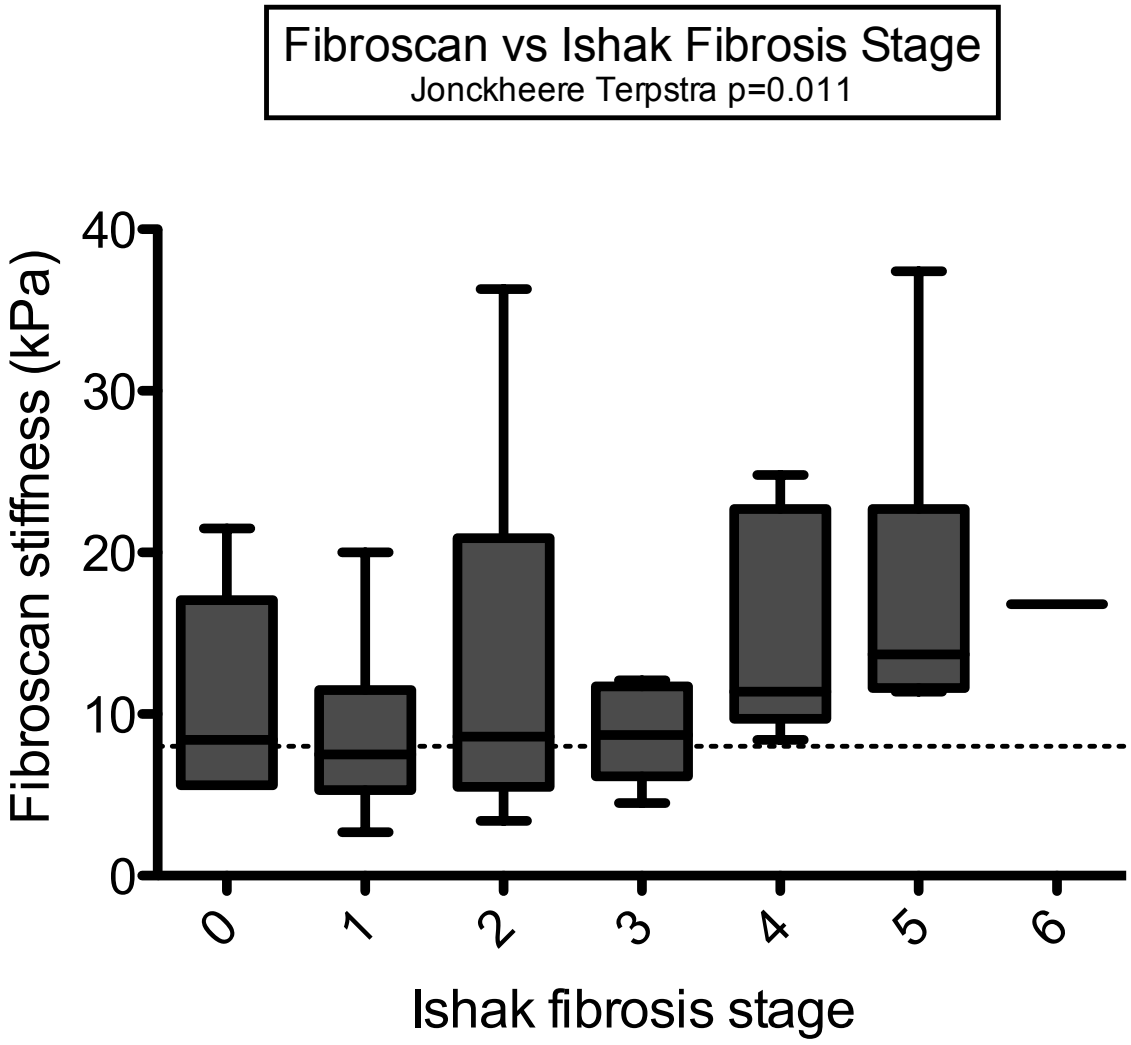


Figure 4-13 Fibroscan readings vs. Ishak fibrosis score with 8kPa cut off plotted

Analysis of Fibroscan data after having excluded the invalid scans reveals a positive correlation between increasing stiffness and fibrosis stage. No patient with cirrhosis had a value <8kPa.

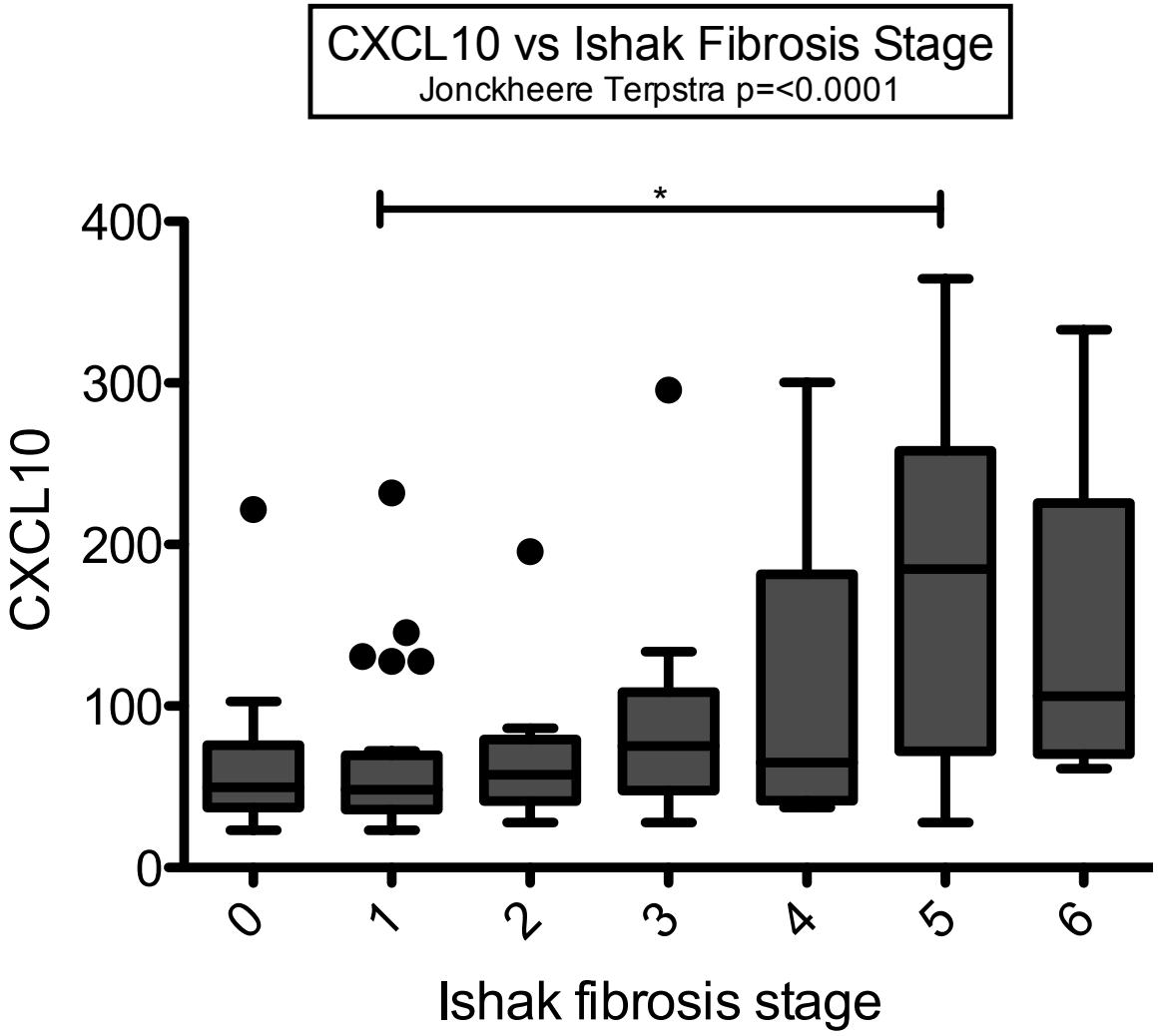


Figure 4-14 CXCL10 (pg/ml) vs. Ishak fibrosis score showing distribution of CXCL10 levels according to fibrosis stage. Pairwise significance seen between F1 and F5

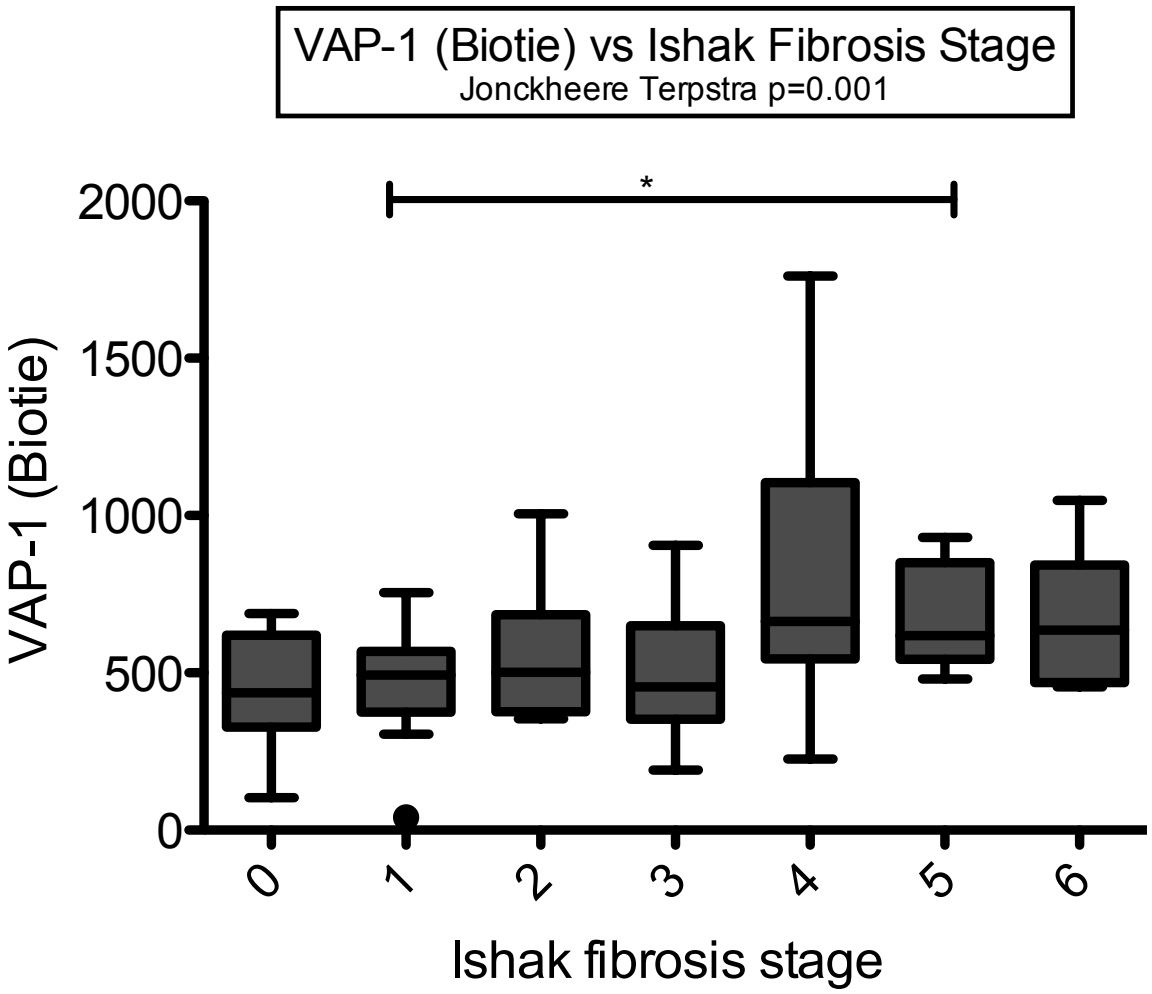


Figure 4-15 Biotie VAP-1 (ng/ml) vs. Ishak fibrosis score showing distribution of VAP-1 levels according to fibrosis stage. Pairwise significance seen between F1 and F5

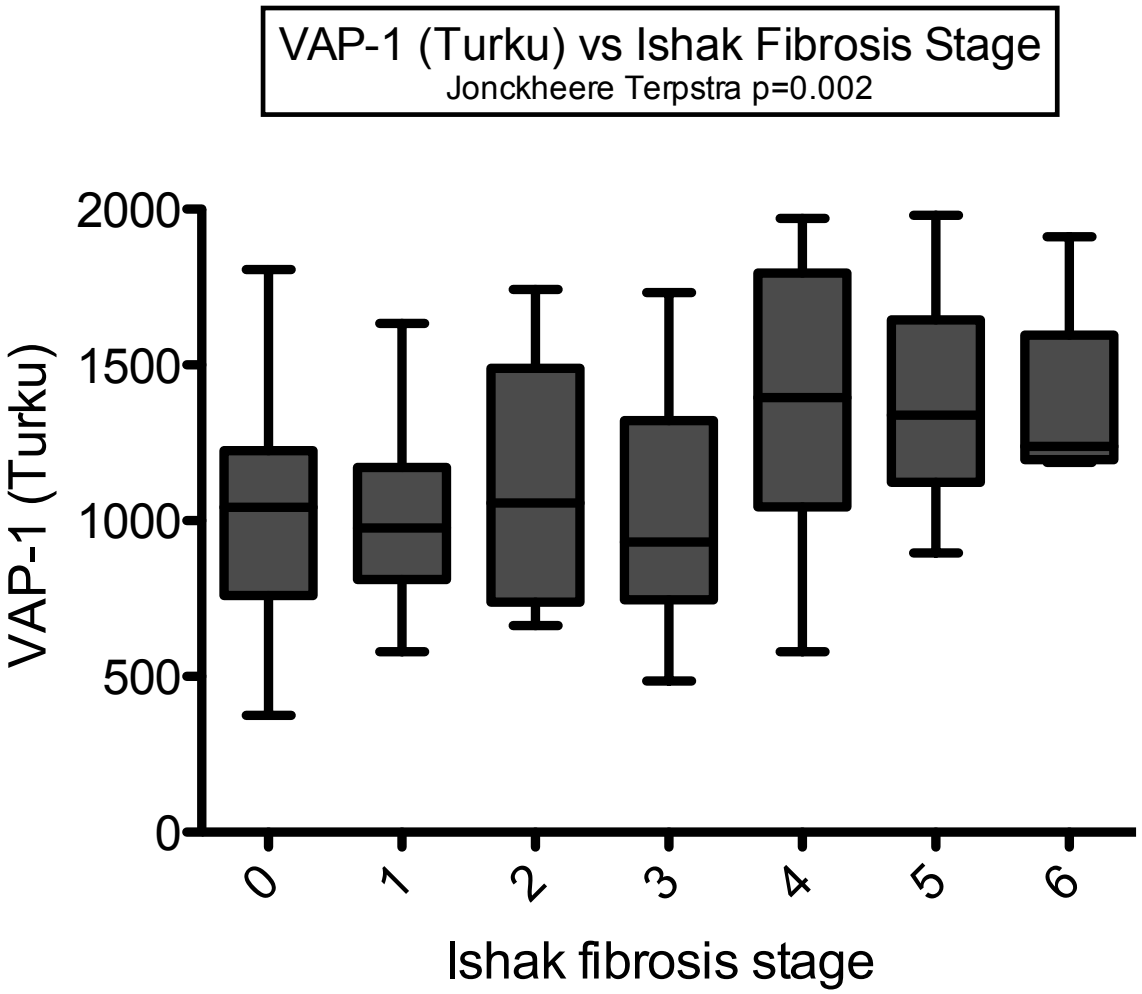


Figure 4-16 Turku VAP-1 (ng/ml) vs. Ishak fibrosis score showing distribution of VAP-1 levels according to fibrosis stage.

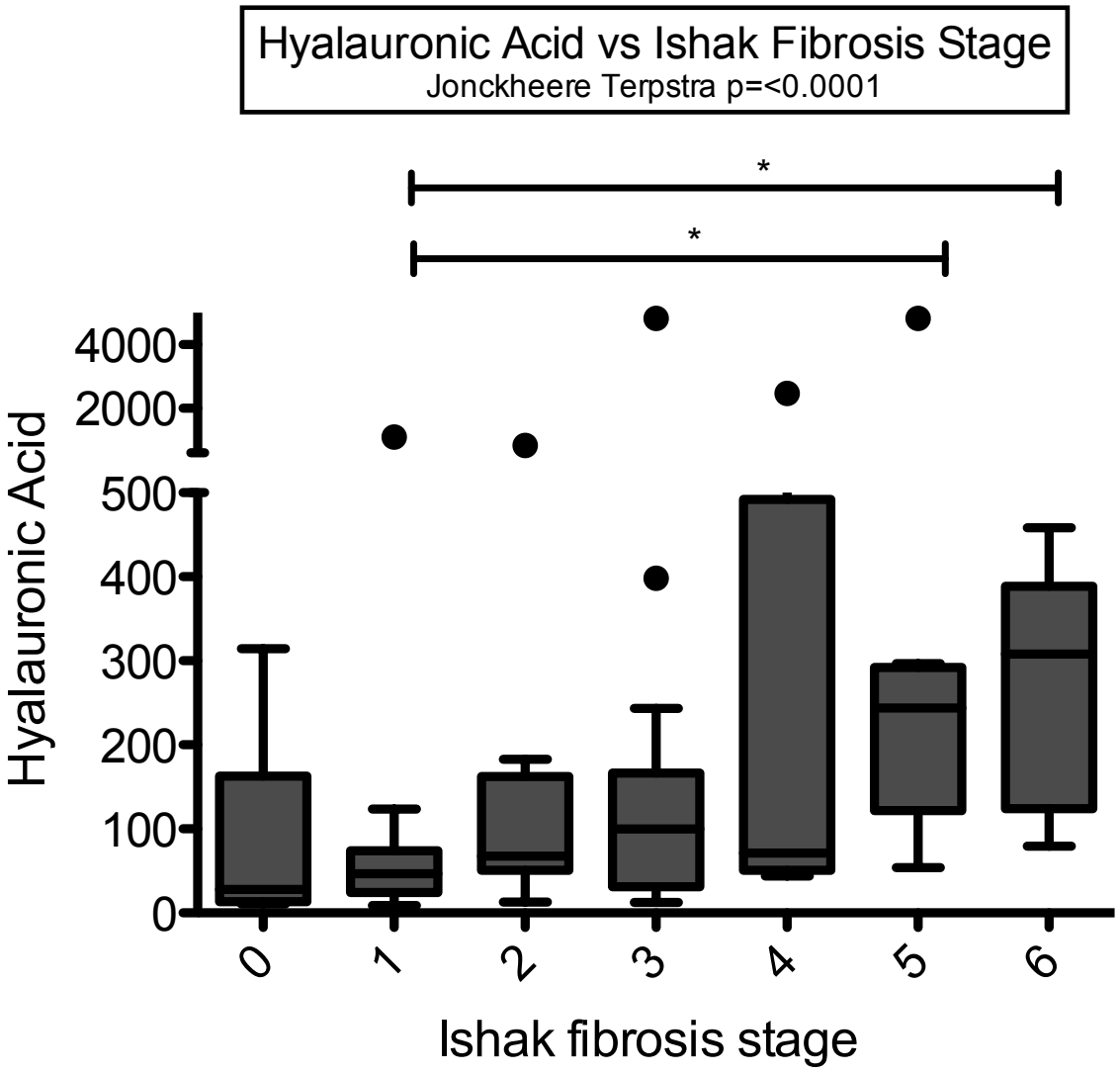


Figure 4-17 Hyalauronic Acid (ng/ml) vs. Ishak fibrosis score showing distribution of hyalauronic readings according to fibrosis stage. Pairwise significance seen between F1 and F5 / F6

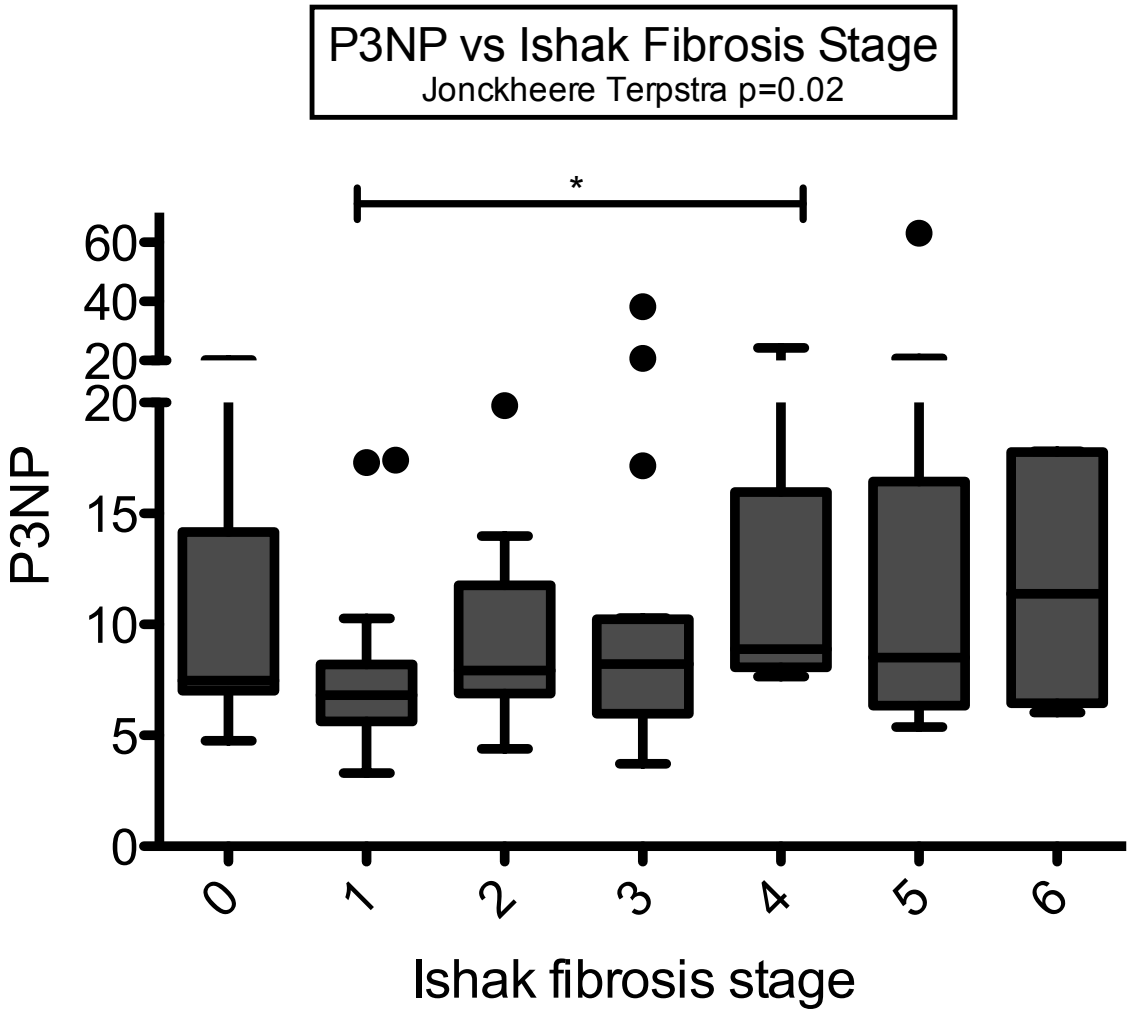


Figure 4-18 P3NP (ng/ml) vs. Ishak fibrosis score showing distribution of P3NP readings according to fibrosis stage. Pairwsie significance seen between F1 and F4

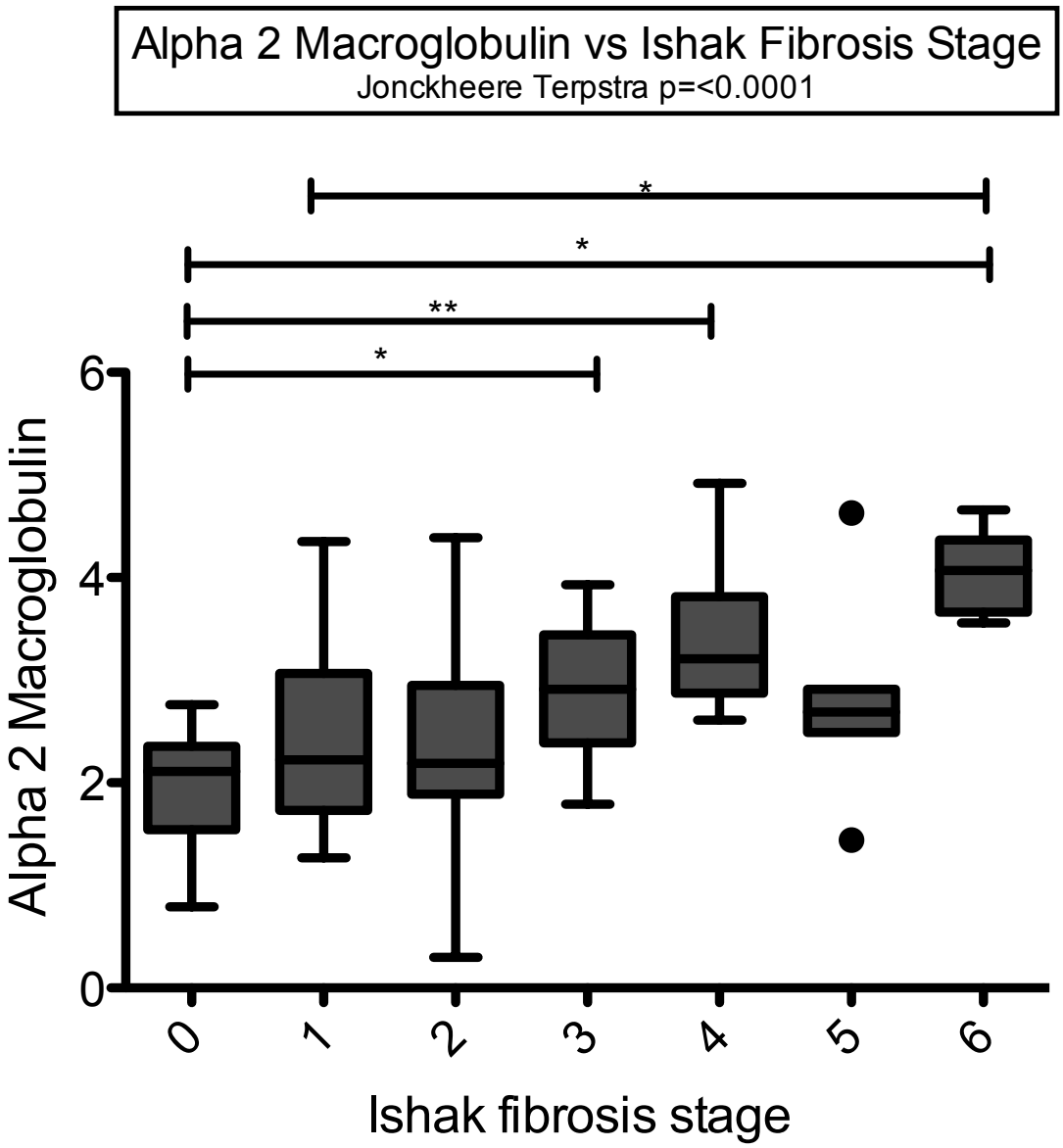


Figure 4-19 Alpha 2 Macroglobulin (g/l) vs. Ishak fibrosis stage showing distribution of alpha 2 macroglobulin readings according to fibrosis stage

4.4.5 AUROC analysis

Test	Ishak 0 vs. 1-6	Ishak 0&1 vs. 2-6	Ishak 0-2 vs. 3-6	Ishak 0-3 vs. 4-6	Ishak 0-4 vs. 5-6	Ishak 0-5 vs. 6
Forns	0.578	0.626	0.622	0.741	0.794	0.846
Fib-4	0.600	0.666	0.681	0.781	0.819	0.842
APRI	0.554	0.651	0.634	0.691	0.696	0.714
AST:ALT	0.638	0.577	0.617	0.605	0.625	0.561
Hepascore	0.650	0.761	0.771	0.791	0.796	0.836
ELF	0.605	0.750	0.744	0.776	0.794	0.764
Fibrotest	0.675	0.703	0.769	0.788	0.759	0.947
VAP-1 Biotie	0.640	0.679	0.679	0.783	0.746	0.673
Vap-1 Turku	0.554	0.650	0.660	0.771	0.765	0.760
CXCL10	0.575	0.677	0.712	0.718	0.782	0.738
Alpha -2 Macroglobulin	0.704	0.728	0.788	0.786	0.719	0.917
Fibroscan	0.513	0.667	0.704	0.808	0.821	0.818
VAP-1 Biotie and CXCL10			0.739			

Table 4-8 AUROC analysis for various markers used in order to predict degrees of liver fibrosis.

4.4.6 Sensitivity and Specificity

AUROC values for each test for the prediction of cirrhosis (F5-6) or significant fibrosis (F3-6) were generated. Using the coordinates of the AUROC curve, sensitivities, specificities, PPV and NPV for differing cut offs were generated. The results are shown in table 4-8.

Test	Cut off	Sens	Spec	PPV	NPV
Hepascore F5-6 AUROC 0.796	0.50	90.9	45.8	0.28	0.96
	0.60	90	58.3	0.33	0.96
	0.80	81.8	66.6	0.36	0.94
Hepascore F3-6 AUROC 0.771	0.50	82.8	60.0	0.67	0.78
	0.60	72.4	70.0	0.67	0.78
	0.80	62.1	76.7	0.72	0.68
CXCL10 F5-6 AUROC 0.782	60	92.9	54.9	0.29	0.98
	100	64.3	77.5	0.36	0.92
	140	50.0	90.1	0.50	0.90
CXCL10 F3-6 AUROC 0.684	60	71.8	63.0	0.62	0.72
	100	43.6	82.6	0.68	0.63
	140	25.6	91.3	0.71	0.59
VAP-1 Biotie F5-6 AUROC 0.746	460	92.9	43.7	0.25	0.97
	590	71.4	74.6	0.36	0.93
	630	50.0	77.5	0.30	0.89
VAP-1 Biotie F3-6 AUROC 0.679	460	74.4	47.8	0.55	0.69
	590	48.7	80.4	0.68	0.65
	630	41.0	84.8	0.68	0.65
ELF F5-6 AUROC 0.794	7.7	100	1.4	0.17	1.00
	9.8	85.7	59.2	0.29	0.95
	12	14.3	94.4	0.33	0.85
ELF F3-6 AUROC 0.744	7.7	97.4	2.2	0.46	0.50
	9.8	69.2	69.6	0.66	0.73
	12	10.3	97.8	0.80	0.56
Fib-4 F5-6 AUROC 0.819	1.5	85.7	58.8	0.30	0.95
	3.25	42.9	92.9	0.55	0.89
Fib-4 F3-6 AUROC 0.681	1.5	64.9	64.4	0.60	0.69
	3.25	18.9	91.1	0.64	0.58
Fibrotest F5-6 AUROC 0.759	0.32	81.8	39.1	0.19	0.93
	0.59	45.5	82.8	0.31	0.90
	0.73	45.5	89.1	0.42	0.90
Fibrotest F3-6 AUROC 0.769	0.32	82.9	52.5	0.60	0.78
	0.59	34.3	90.0	0.75	0.61
	0.73	25.7	92.5	0.75	0.59
Fibroscan F5-6 AUROC 0.821	8	100	39.5	0.23	1.00
	12.5	57.1	84.2	0.36	0.91
Fibroscan F3-6 AUROC 0.704	8	88.9	48.1	0.53	0.87
	12.5	33.3	81.5	0.55	0.65

Table 4-9 Sensitivity, Specificity, Negative and Positive predictive value of tests + cut offs.

4.4.7 Logistic Regression to predict fibrosis

In order to predict significant fibrosis for Ishak 3-6, logistic regression was used in a stepwise manner. The only variables that were significant were VAP-1 (both Biotie and Turku) and CXCL10 or VAP-1 and alpha 2 macroglobulin. Alpha 2 macroglobulin was analysed in the presence of incomplete data (i.e. 77). Data is shown in tables 4-9 and 4-10.

This incomplete number means that there will be less power to detect an effect. The reasons for lack of data were absence of kits available and samples being lost in the post. This equates to a random effect and should not bias the results. Given the small numbers of patients with F5-6 disease, attempting logistic regression would be statistically un-sound(Peduzzi et al., 1996). When entering VAP-1 (biotie) and alpha-2 macroglobulin into stepwise logistic regression, the alpha-2 macroglobulin is significant as is the VAP-1 hence it is entered into the model, but upon analysis, due to the coefficient changing, VAP-1 loses significance. This is hard to interpret.

The Odds ratio (OR) here used with a continuous variable represents the effect of an increase in 1 of the variable. Therefore Alpha 2 Macroglobulin which has very low values is likely to have a greater odds ratio than VAP which has values often in the thousands. To adjust for this, standardised values were created by dividing by the standard deviation (SD) and the OR were recomputed as below (marked “std”): These OR represent an increase in 1SD of the unstandardized variables rather than an absolute value of 1.

Marker	Sig	Odds Ratio	95% CI
CXCL10	0.011	1.010	1.002-1.019
Vap-1 Biotie	0.028	1.003	1.000-1.005
CXCL10	0.013	1.010	1.002-1.018
Vap-1 Turku	0.084	1.001	0.999 -1.003
Alpha 2 Macroglobulin	0.001	3.053	1.571-5.931
Vap-1 Biotie	0.068	1.002	0.999-1.005
Alpha 2 Macroglobulin	0.001	3.249	1.673-6.309
Vap-1 Turku	0.114	1.001	0.999-1.002

Table 4-10 Analysis for F0-2 vs. 3-6 using non standardized values

Marker	Sig	Odds Ratio	95% CI
CXCL10 std	0.011	2.252	1.200-4.225
Vap-1 Biotie std	0.028	2.048	1.080-3.880
CXCL10 std	0.013	2.155	1.177-3.949
Vap-1 Turku std	0.084	1.573	0.942-2.626
Alpha 2 Macroglobulin std	0.001	2.878	1.534-5.397
Vap-1 Biotie std	0.068	1.797	0.958-3.369
Alpha 2 Macroglobulin std	0.001	3.052	1.628-5.722
Vap-1 Turku std	0.114	1.508	0.906-2.512

Table 4-11 Analysis for F0-2 vs. 3-6 using standardized values

4.4.7.1 Logistic regression to predict fibrosis – improving on commercial markers

As above in order to predict significant fibrosis for Ishak 3-6, logistic regression was used in a stepwise manner but using commercial markers where all variables were entered into the model whether they were significant or not. 3 models were used: VAP1, CXCL10 and then one of ELF, Hepascore or Fibrotest.

Once ELF has been included in the model, neither VAP-1 (p=0.100) nor CXCL10 (p=0.094) adds significantly to the model.

Once Hepascore has been included in the model, neither VAP-1 (p=0.119) nor CXCL10 (p=0.213) adds significantly to the model.

Once Fibrotest has been included in the model, VAP-1 (p=0.159) does not add significantly to the model but CXCL10 (p=0.032) does add significantly to the model.

Once Fibrotest and CXCL10 have been included in the model, VAP-1 (p=0.279) does not however add significantly to the model.

4.4.8 Mann Whitney U analysis for comparison of Ishak stage 0-2 vs. 3-6

Mann Whitney U testing is a non-parametric test to test a null hypothesis that two populations are the same against an alternative hypothesis. It provides greater efficiency than a t-test on non-normal distributed data hence its application here.

Significant values in this analysis ($p<0.05$) show that the value of the variable in question is significantly different between the two grouped Ishak stages.

Test	Mann-Whitney U	Ishak stage 0-2 median ±SD	Ishak stage 3-6 median ±SD	Sig	N if <85
Forns	351	7.91±1.6	8.47±2.0	0.103	61
Fib-4	531	1.19± 1.02	1.69 ± 1.76	0.005	82
APRI	609	0.43±0.82	0.58±0.73	0.037	82
AST:ALT	638	0.81±0.33	0.93±0.73	0.07	82
Hepascore	200	0.35± 0.32	0.86± 0.26	0.001	59
Fibrotest	323.5	0.29± 0.22	0.53±0.25	<0.0001	75
ELF	459.5	9.29±1.07	10.13± 1.60	<0.0001	
Fibroscan	144	8.40 ±7.24	11.65 ±7.68	0.022	45
CXCL10	517	51.93± 49.39	82.88± 93.43	0.001	
VAP-1 Biotie	575	468.0± 172.28	582.0± 301.02	0.005	
VAP-1 Turku	610	1025.0± 316.75	1187.0± 399.75	0.011	
HA	463	51.35± 196.82	138.15± 1099.4	<0.0001	
P3NP	619.5	7.22± 4.11	8.59±10.68	0.014	
TIMP1	679.5	246.1±71.82	296.7± 177.98	0.055	
Alpha 2 Macroglobulin	313.5	2.19± 0.87	3.06± 0.81	<0.0001	77
Haptoglobin	541.5	1.60± 0.60	1.28± 0.58	0.133	74
Apolipoprotein A1	635.5	1.51± 0.26	1.47± 0.42	0.295	77

Table 4-12 Mann Whitney I analysis for comparison of Ishak stage 0-2 vs. 3-6

4.4.9 Comparison of Biotie sVAP and Turku sVAP

The raw data is plotted below.

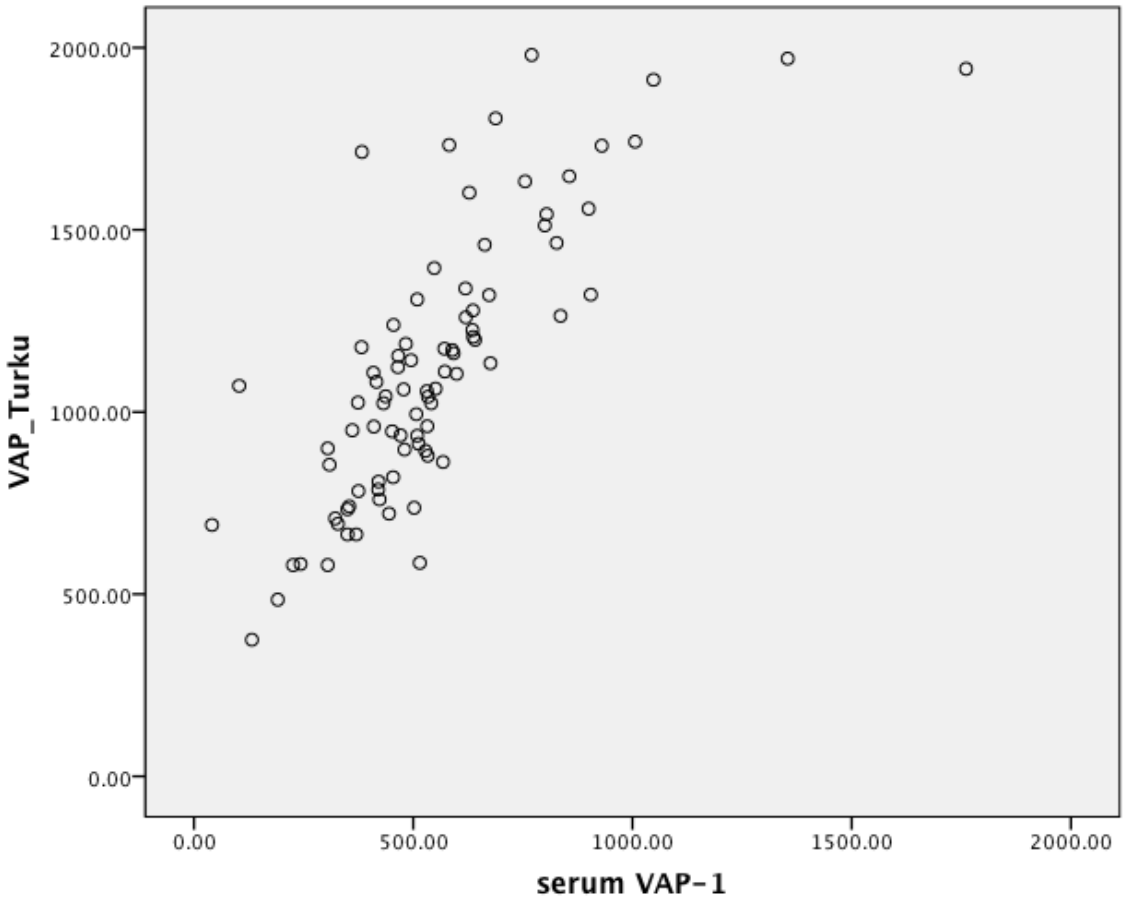


Figure 4-20 VAP-1 (Turku lab) vs. VAP-1 (Biotie lab). Both measured in ng/ml

There was a good correlation between the two values. Pearson correlation was 0.784 ($p<0.0001$) and Spearman was 0.801 ($p<0.0001$)

The plot below (figure 4-21) shows a Bland –Altman plot for untransformed data. The difference tends to increase with the mean

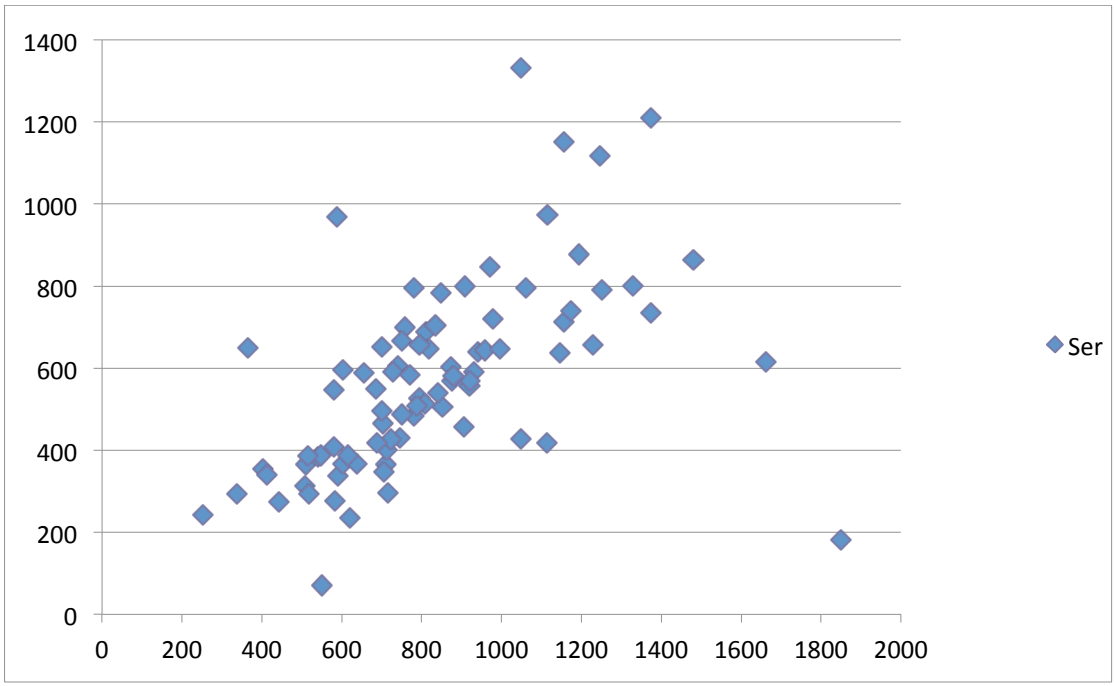


Figure 4-21 Bland-Altman plot of untransformed VAP-1 (Biotie) vs. VAP-1 (Turku). Both measured in ng/ml

If the data is logged and plotted again on a bland-altman plot (figure 4-22), this difference with mean is lost suggesting a proportional bias. The values from Turku are approximately twice that of the Biotie, which is supported by the third plot (figure 4-23), which illustrates the ratio between the two data sets.

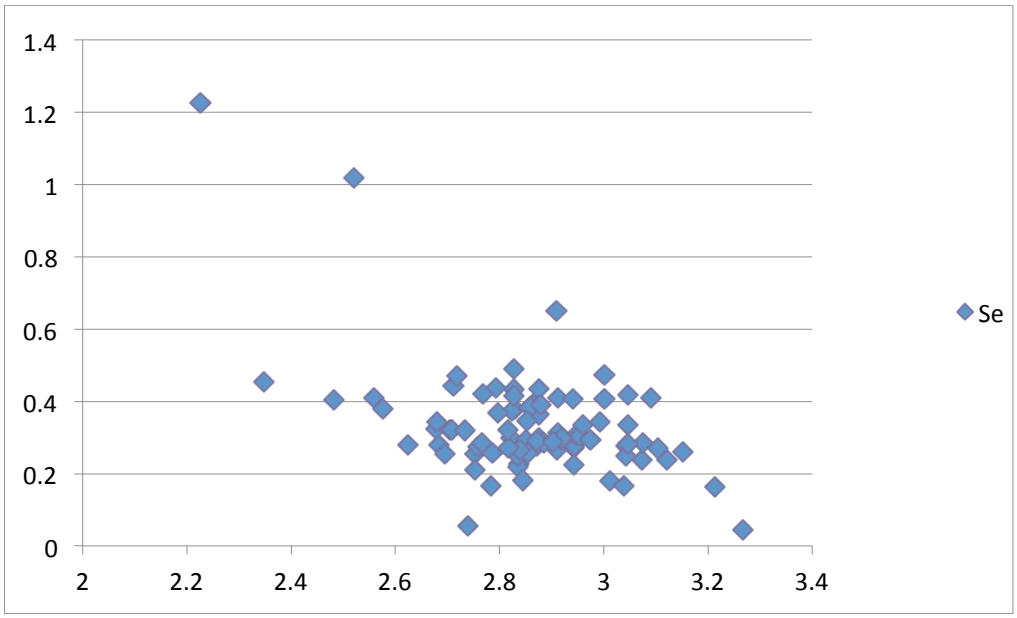


Figure 4-22 Bland-Altman plot of logged VAP-1 (Biotie) vs. VAP-1 (Turku) showing a proportional bias between the two measured levels

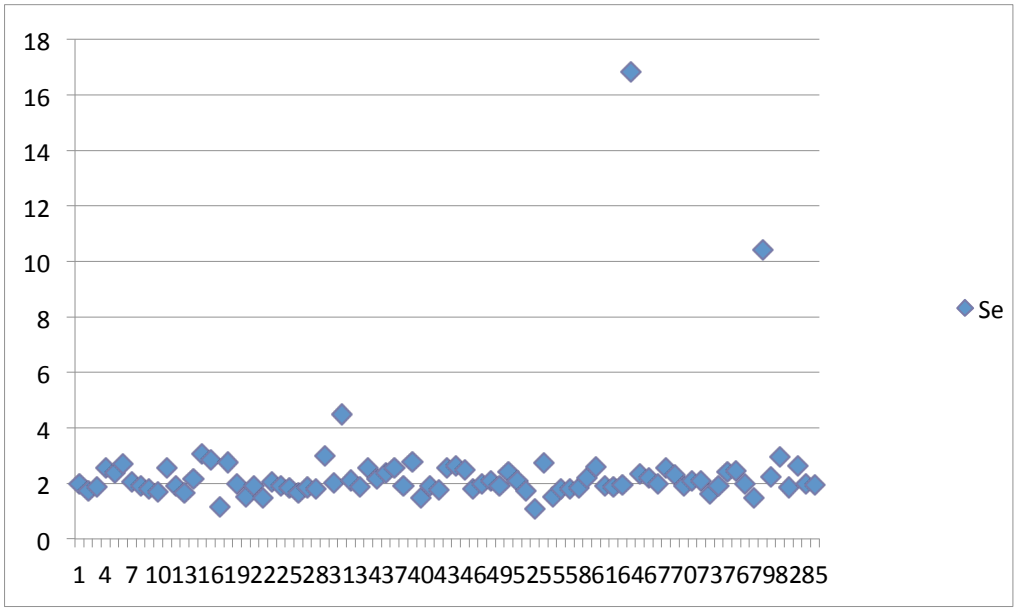


Figure 4-23 Ratio between VAP-1 (Biotie) and VAP-1 (Turku) showing a constant ratio between the two measurements

4.4.10 Image J analysis

4.4.10.1 Standardised Image J analysis

78 slides were available for analysis as some blocks could not be located and others were not available due to use in other research projects. This was a random effect again and should not bias the result.

Standardised values for image hue, saturation and brightness were used.

Pearson correlation between Image J values and Ishak stage were poor at 0.400 ($p<0.0001$), but when personalised settings were used, the value increased markedly to 0.810 ($p<0.0001$). Spearman correlations were similar (0.329 and 0.829 respectively).

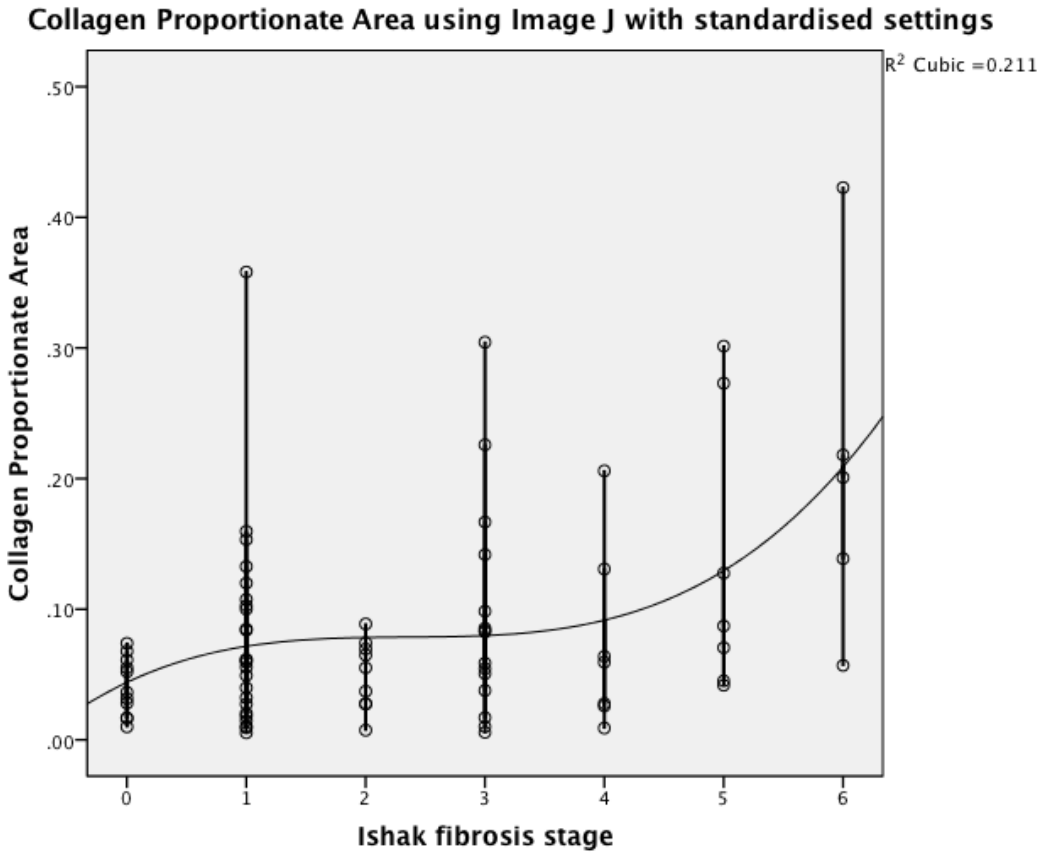


Figure 4-24 Collagen proportionate area using Image J with standardised settings

The table below reports the mean and median collagen proportionate areas when standardized settings are used.

Ishak fibrosis stage	Mean	N	Std. Deviation	Median
0	.040879	11	.0222750	.036345
1	.077798	24	.0756007	.060307
2	.050245	9	.0267095	.055176
3	.094889	15	.0837261	.082509
4	.074674	7	.0702391	.059483
5	.135242	7	.1080090	.087191
6	.207490	5	.1359339	.200842
Total	.085888	78	.0839097	.060325

Table 4-13 Image J CPA mean values compared to Ishak fibrosis stage

4.4.10.2 Personalized Image J

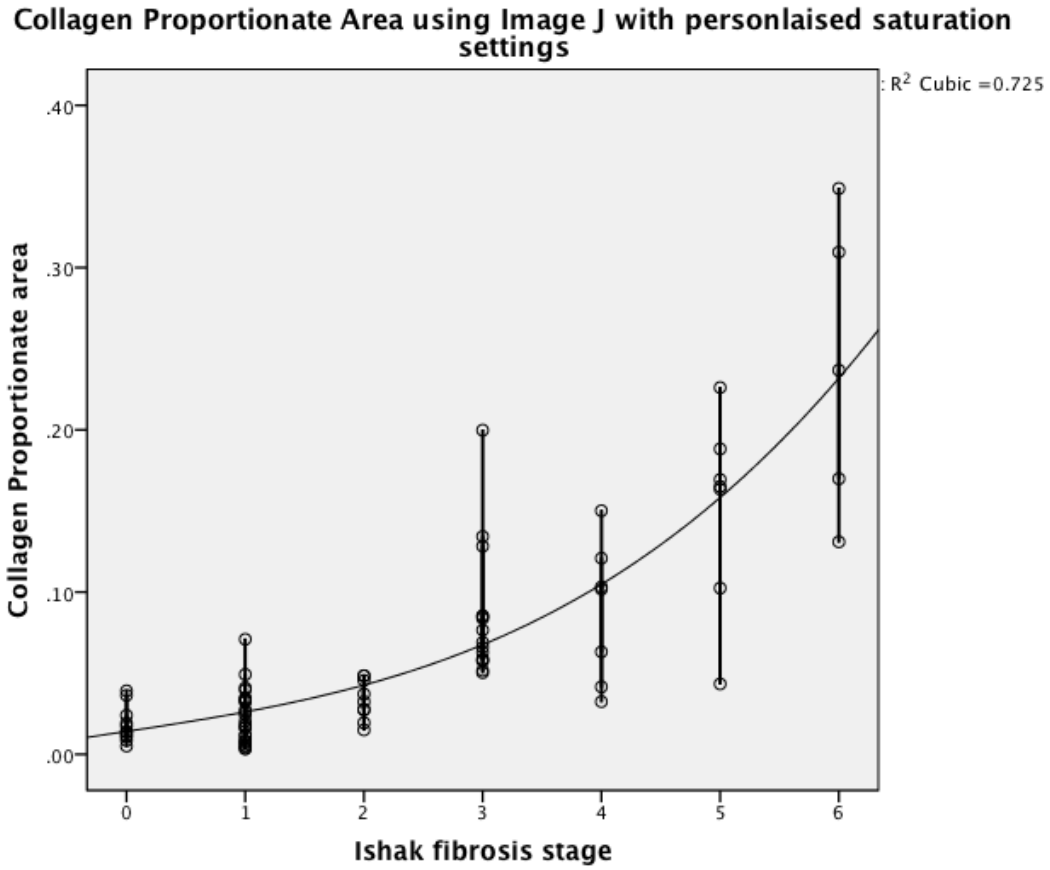


Figure 4-25 Collagen proportionate area using Image J with standardised settings

The table below shows the mean and median personalized saturation settings used during the personalized analysis

Ishak fibrosis stage	Mean	N	Std. Deviation	Median
0	81.36	11	9.179	80.00
1	76.92	26	25.580	82.50
2	75.11	9	8.810	75.00
3	63.44	16	20.633	67.50
4	44.67	9	32.334	55.00
5	40.11	9	31.267	51.00
6	54.00	5	21.909	60.00
Total	66.11	85	26.661	70.00

Table 4-14 Personalised Image J settings showing mean saturation settings used.

Table 4-15 shows the collagen proportionate area results (mean and median) when personalized settings are used.

Ishak fibrosis stage	Mean	N	Std. Deviation	Median
0	.018152	11	.0109808	.013880
1	.022858	24	.0168104	.019255
2	.033504	9	.0123297	.032456
3	.084487	15	.0406477	.069008
4	.087611	7	.0432270	.101778
5	.151150	7	.0600619	.165079
6	.239182	5	.0914836	.236777
Total	.066466	78	.0708790	.040320

Table 4-15 Collagen proportionate area results using personalised settings showing a rise in mean CPA with fibrosis stage

The R squared result for the standardized results was 0.160 and 0.656 for the personalized settings.

When the difference in saturation settings used from the baseline of 70, a clear pattern is seen. At lower levels of Ishak fibrosis stage, Image J with standardized settings over-reads collagen hence the saturation level has to be increased to account for this. Ishak stage 3 is where this crosses over and Image J begins to under-read hence the need to decrease the saturation settings.

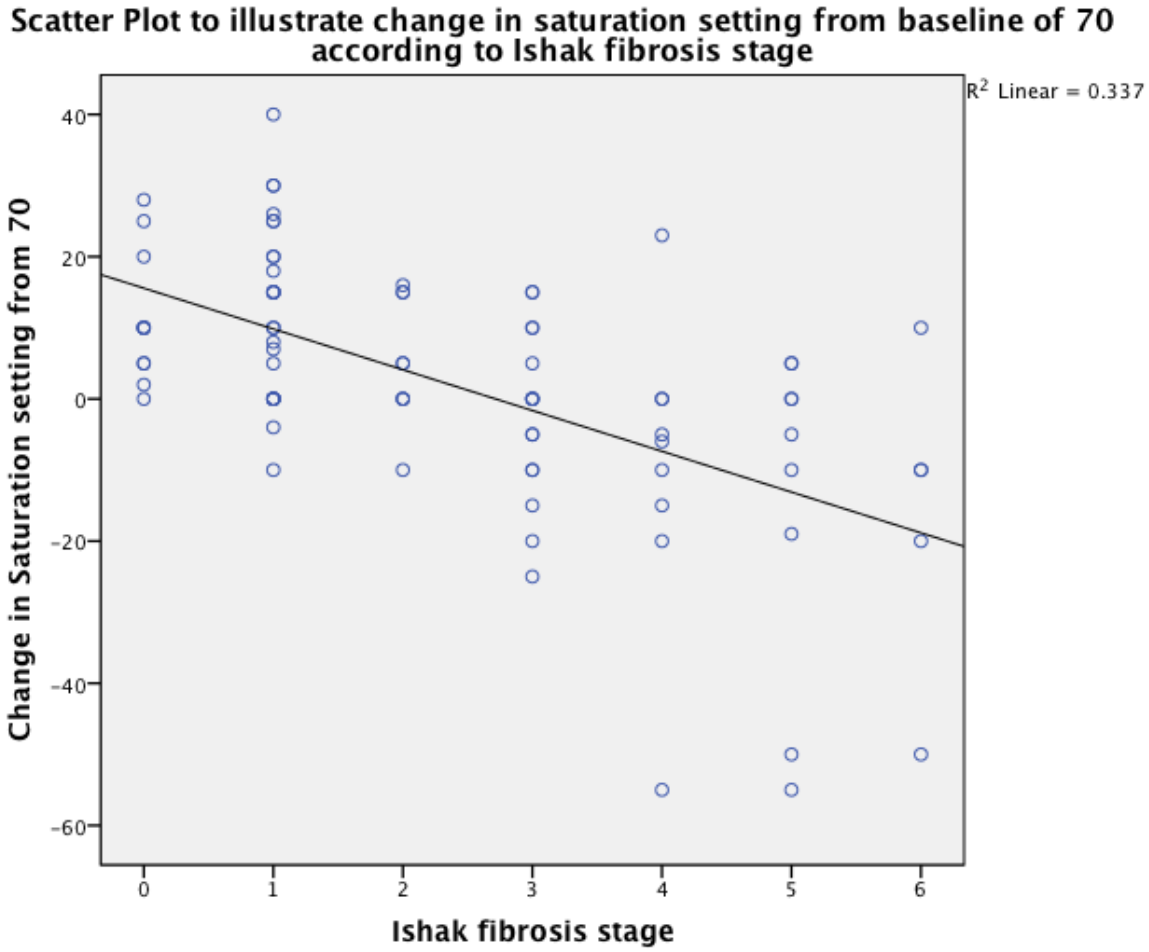


Figure 4-26 Scatter plot to illustrate change in saturation settings according to Ishak stage

4.4.11 Using the personalized Image J settings as an alternative gold standard

	Spearman	Significance	N if<78
Fib-4	0.359	0.002	75
APRI	0.228	0.049	75
VAP-1 Biotie	0.302	0.007	
VAP-1 Turku	0.276	0.015	
HA	0.478	<0.0001	
P3NP	0.355	0.001	
TIMP1	0.325	0.004	
CXCL10	0.455	<0.0001	
Alpha 2 Macroglobulin	0.601	<0.0001	73
Haptoglobin	-0.188	0.119	70
Apolipoprotein A1	-0.042	0.726	73
Fibrotest	0.482	<0.0001	71
Forns	0.349	0.008	56
AST:ALT	0.238	0.049	75
Fibroscan	0.451	0.003	41 (45 valid)
ELF	0.510	<0.0001	
Hepascore	0.563	<0.0001	55
VAP-1 and CXCL10	0.443	<0.0001	

Table 4-16 Correlation of variables to Image J results as alternative standard to Ishak

4.4.11.1 AUROC analysis of Image J

To predict significant fibrosis (Ishak stages 3-6), the personalised Image J settings had an AUROC value of 0.768, which dropped to 0.551 when the standardised settings were used.

4.4.12 Image J VAP-1 analysis

There was no correlation between VAP-1 staining and Ishak fibrosis stage (figure 4-27). There was also no correlation between serum VAP-1 levels and VAP-1 staining (Spearman correlation -0.212 P=0.556).

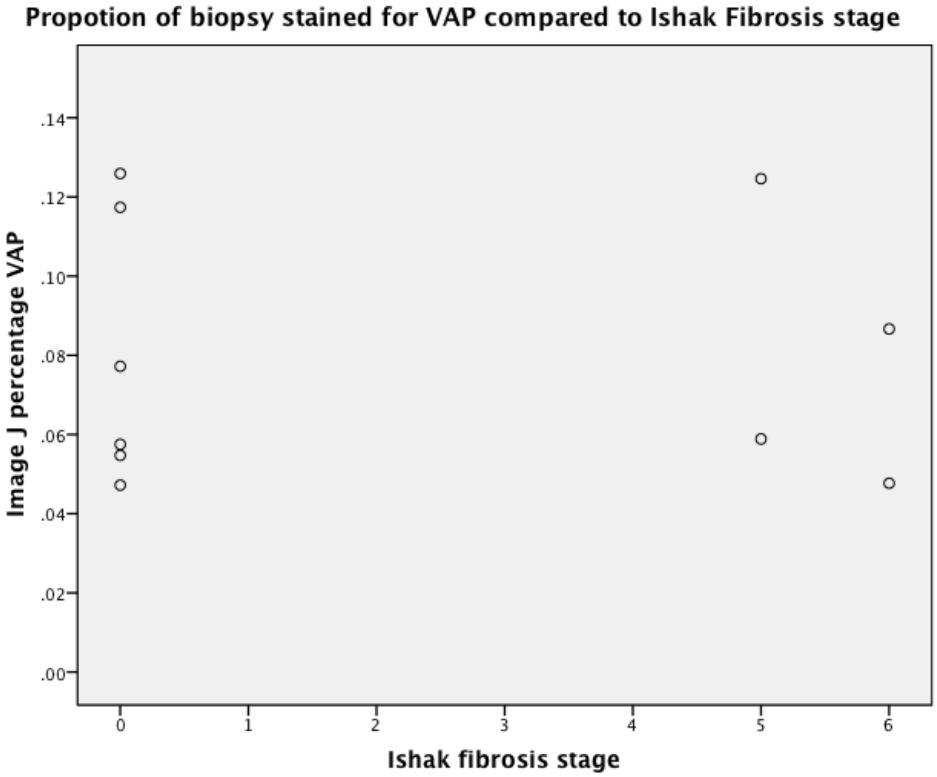


Figure 4-27 Scatter plot of VAP-1 staining vs. Ishak fibrosis stage showing no correlation

4.4.13 SFLC and Cystatin C analysis

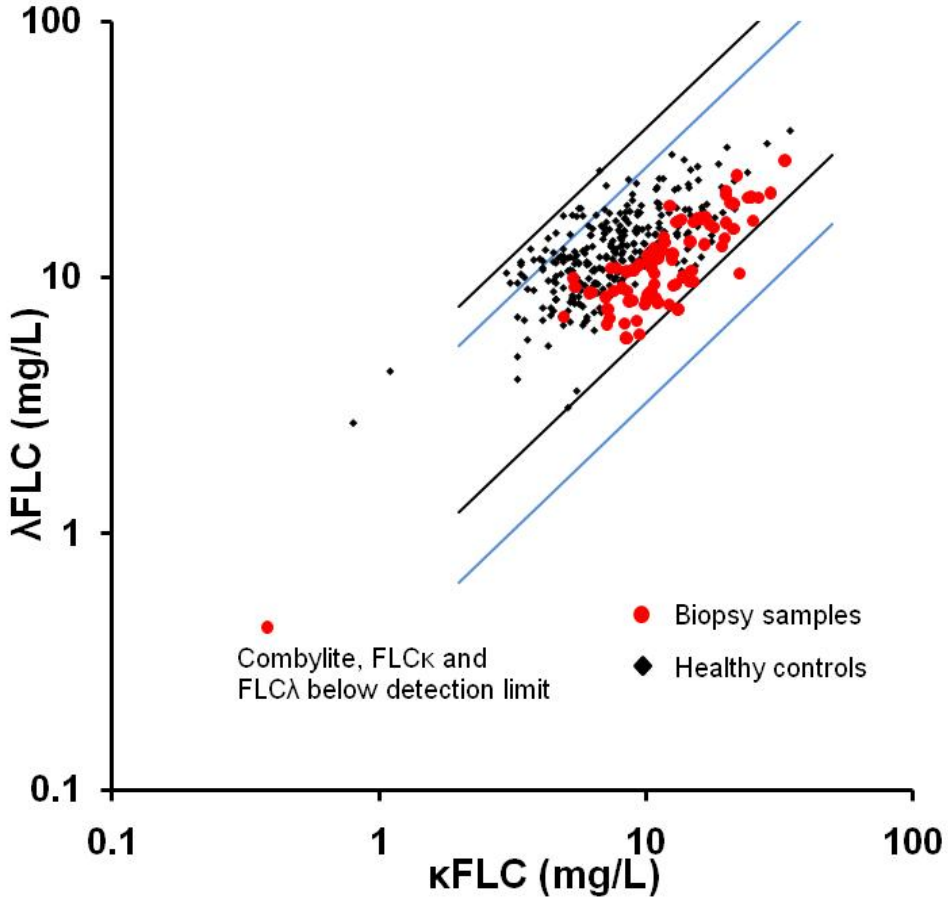


Figure 4-28 Scatter graph of Freelite kappa and Freelite lambda. Samples from liver biopsy samples (red dots) showed a slight increase in light chain concentrations and a shift towards kappa ratios compared with healthy controls (black diamonds). One sample showed Combylite and Freelite concentrations below the assay detection limits

This is a scatter graph of Freelite kappa and Freelite lambda. Samples from the study (red dots) showed a slight increase in light chain concentrations and a shift towards kappa ratios compared with healthy controls (black diamonds). One sample showed Combylite and Freelite concentrations below the assay detection limits. Any monoclonal FLC expansions are shown by kappa/lambda ratios that fall outside a normal range of 0.26-1.65. Patients with renal insufficiency show increased FLC concentrations and a shift toward kappa ratios due to

the reduced FLC clearance, and there is a defined reference range for patient with renal failure (0.37-3.1, blue lines)

The median Combylite concentration in samples was 25.2mg/L, which is higher than in healthy controls (20mg/L). Seven patients showed Combylite levels >50mg/L, which approximates to the summation of the individual reference ranges for FLC kappa and FLC lambda assays. Combylite levels are modestly increased in the liver biopsy samples compared with healthy controls, with highest levels in viral samples.

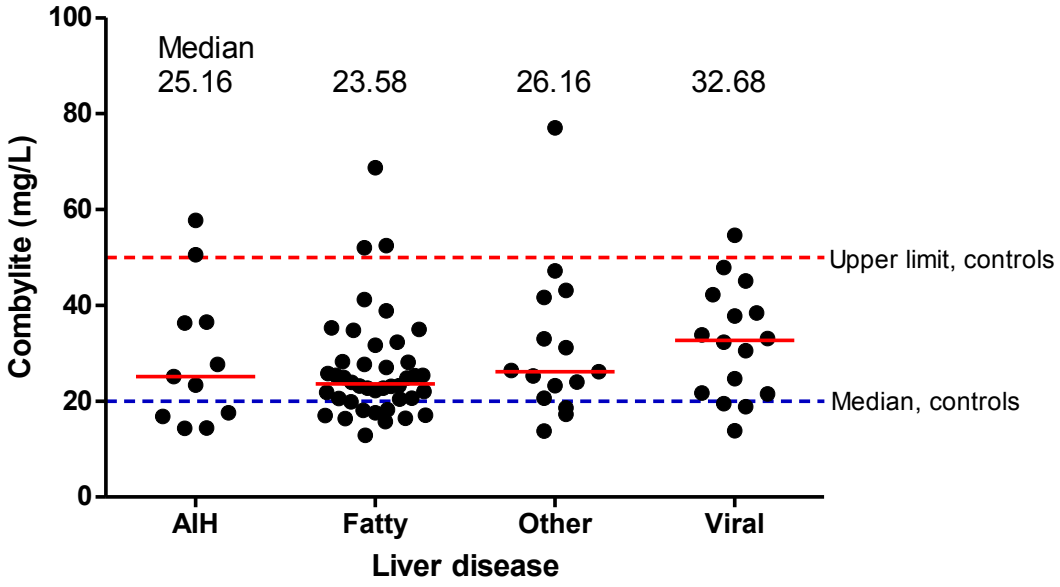


Figure 4-29 Combylite concentrations by liver disease diagnosis

4.5 Discussion

Establishing a reproducible, accurate and cheap non-invasive marker for liver fibrosis has been the aim of researchers for years. Research into the field has been progressing recently with many studies adding weight to earlier findings and validating them. However commercially available panels have not been widely adopted into clinical practice for several reasons. Any new research that introduces new clinical tests must be repeatedly validated before it will be accepted into practice. This is especially so when decisions made upon the information provided could have consequences to patient care. In order to validate new findings, samples have to be prospectively collected an in large numbers with multiple centres. This all takes time but is necessary to quash criticisms. Such studies are also expensive to run.

Small studies like ours are valuable because they provide further validation for existing biomarkers but also s help to identify potential new markers based upon on going research that may be added to the panels in the future. This study does have several limitations. It is a single centre study and has no validation cohort. Numbers included are therefore relatively small. However the fact that our study shows ROC values for ELF and Fibrotest that are comparable to previous studies suggests the findings are valid.

The study is prospectively collected cohort with no selection basis other than that introduced by the requesting clinician. It is perhaps not surprising that there is a high proportion of low to middle grade Ishak stage samples, as clinicians are unlikely to biopsy cirrhotic patients because the information would not aid them in day-to-day practice.

Patients were not selected for biopsy on the basis of a research question as is done in many other studies but represent a valid representation of every-day patients put forward for biopsy.

There was a high rate of Fibroscan invalidity (42%). This is much greater than in published studies of Fibroscan. The high proportion of fatty liver patients can explain this high rate. In normal practice, the clinician may decide against a scan due to BMI but in the study, these patients were scanned regardless. Many clinical trials using Fibroscan are performed in viral hepatitis patients who often have a lower BMI than the fatty liver patients. It is reassuring that no patient with a Fibroscan result <8kPa had a histological diagnosis of Ishak stage ≥4. It also validates the findings of our earlier study that the Fibroscan should be used to rule out disease rather than to rule it in (Corbett et al., 2013). In neither study did any patient with F5-6 disease have a scan measurement of <8kPa. Correlations are also far more significant when invalid scans are excluded, re-affirming the importance of validity. It is reassuring that similar findings are seen when both retrospective and prospective data collection methods are used.

4.5.1 CXCL10 and VAP-1

We have focussed particularly on two potential biomarkers, which our laboratory has shown in the past to be essential in the development of chronic hepatitis in clinical studies and animal models of liver disease CXCL10 and VAP-1. Based on their biological function and strong mechanistic association with the development of chronic hepatitis and liver injury we predicted they would be good biomarkers of liver fibrosis. This was indeed the case and both are independently associated with fibrosis in our study. They can be paired in logistic

regression to improve the predictive ability of each marker to predict significant fibrosis. Given the small numbers (<20 in F5-6) it would have been statistically un-sound to try the same for F5-6. The ROC value created (0.739) by combining VAP-1 and CXCL10 through logistic regression to make a predicted probability for Ishak 0-2 vs. 3-6 is similar to that seen with commercially available tests.

Our study confirms the link between VAP-1 and fibrosis and this is consistent with work currently submitted for publication form our group reporting that VAP-1 plays an important role in the development of hepatitis and fibrosis in several animal models of liver disease. In order to assess its clinical use, larger prospective studies are required where we can assess serial measurements and see how well it predicts clinical events and outcome. We measured sVAP-1 in two different collaborating laboratories and although the patterns of expression were the same the absolute values showed some differences emphasising the importance of establishing reliable clinically validated assays before this test can be used clinically.

4.5.2 VAP-1 and alpha-2 macroglobulin

Logistic regression analysis of these two markers yielded an un-interpretable result. Stepwise analysis revealed that both markers could be entered into the model, but that once the model was run, due to the change in co-efficient, the significance of adding VAP-1 was lost. This is difficult to explain and is probably a function of small numbers.

4.5.3 Collagen proportionate analysis (CPA)

The accuracy of CPA to measure fibrosis depends on biopsy length according to a recent paper(Hall et al., 2013) with best accuracy seen in biopsies of 22-28mm. The median length of the biopsies in the study was 23.5mm. Only 5 biopsies were ≤15mm and only 1 was ≤10mm. What became clear from morphometric analysis was that a "one size fits all" approach with the same setting applied to each slide with CPA is not accurate. The correlations between the standardised settings applied to each individual slide with Ishak staging were far worse than when personalised settings were applied to slides. The weakness in comparing a histological score and a morphometric analysis is the fact that the morphometric analysis gives a linear scale with presumed equal rise in fibrosis between Ishak stages whereas Ishak is not however a linear scale and encompasses much more than just collagen load. The scale was invented to not only describe fibrosis but also structural changes present to the trained eye of the histopathologist.

4.5.3.1 Comparing standardised to personalised Image J settings

As explained above, each slide was analysed suing standardised Image J settings. It was then re-examined, using a saturation setting that was determined by the analyst. Sometimes this was the same as the standard setting, but usually differed especially towards either end of the Ishak spectrum (see Figure 4-26 Scatter plot to illustrate change in saturation settings according to Ishak stage). The proportions of collagen within a biopsy have been analysed in previous papers (Standish et al., 2006) showing the non-linear nature of the rise in collagen. The table below shows the comparison between the mean CPA of the standardised settings

vs. the personalised settings. Clearly the personalised settings increase throughout the Ishak stages rather than having no pattern other than a marked increase in fibrosis at Ishak stage 6 (established cirrhosis) when standardised results are used. The personalised results also compare favourably to the Standish paper. Spearman correlation of the personalised settings to the Standish results is 0.984 compared to 0.795 with standardised settings. The two settings do correlate with each other (0.880).

Ishak Stage	Standardised Image J result (% collagen)	Personalised Image J result (% collagen)	Standish paper mean result
0	3.6%	1.3%	1.9%
1	6.0%	1.9%	3.0%
2	5.5%	3.2%	3.6%
3	8.2%	6.9%	6.5%
4	5.9%	10.1%	13.7%
5	8.7%	16.5%	24.3%
6	20.0%	23.6%	27.8%

Table 4-17 Values of CPA compared to Ishak stage having used standardised and personalised settings. Comparison to Standish results included

The weakness of using personalised settings in the analysis of the slides is the potential to introduce bias. A truly automated system without human interference can be relied upon to make decisions based upon a protocol. Until such a system is developed, the potential for bias exists. In this case the results are far more representative of previous published data.

4.5.3.2 Slide Staining

There also remains the unanswered question over the optimal stain to be used. We used Van Giessen as this is the standard stain used for clinical analysis in our histopathology laboratory but others have used a Sirius Red stain. In order to allow Image J to pick out contrasting colours, it was necessary to use the stain without a counter stain. This meant

that the slide could only be used for the purpose of morphometric analysis and not for other diagnostic reasons. This may have implications when a small block is present such as might be the case with trans-jugular liver biopsies. Morphometric analysis is also very time demanding and until some of the process can be automated, it will remain largely a research tool. The problem with automating the process is that as I have demonstrated, standardised values are inappropriate for analysis as error is introduced at the extremes of Ishak staging. Until a reliable automated process can be developed that accounts for this its use will be limited.

4.5.3.3 VAP Morphometric analysis

As can be seen from the morphometric analysis of the VAP-1 staining, there was no correlation between serum levels of VAP-1 and the degree of staining for VAP-1 on the liver tissue. From work done by our lab we do know that total serum VAP-1 concentration correlates well with serum VAP enzyme activity (Kurkijärvi et al., 1998) and recent unpublished work (Trivedi et al., 2013). The analysis was done using explanted liver, but as far as the authors are aware, there are no published data comparing tissue and serum levels of VAP-1. Most of the staining was sinusoidal and on the vessels as has been previously described and it is possible that this distribution does not give a large enough stained area for the morphometric analysis to be sensitive enough. Perhaps a better way of analysing VAP-1 levels in tissue would be a western blot which would also allow analysis of enzyme activity but this was beyond the remit of the current project.

4.5.3.4 Use of CXCL-10 ± VAP-1 to composite panels for fibrosis testing

There is the potential in the future to add CXCL-10 and VAP-1 to composite panels such as ELF and Fibrotest to increase their accuracy in predicting liver fibrosis. Our data has shown that CXCL10 can be added to Fibrotest to improve its performance. No other result was significant although adding CXCL10 to ELF approached significance. Clearly this is promising, but needs to be confirmed in larger studies and to confirm its clinical utility.

4.6 Limitations

The small number of samples included limits a study like this. Given the amount of tests that were done it is possible that one of them may have been positive by chance. However, no test was done without justification, which is detailed earlier. In order to improve on the statistical power of this study, it needs more numbers, and hopefully colleagues upon my finishing at the University will carry on this study.

4.7 Conclusion

This study has confirmed that commercially available non-invasive biomarkers (ELF and Fibrotest) and most of their constituents correlated with fibrosis stage on liver biopsy.

Our study is novel because it analysed such a large panel of immune and fibrosis markers in patients undergoing liver biopsy and Fibroscan and because it reports correlations between VAP-1 and CXCL-10, new biomarkers with histological fibrosis.

The novel finding that CXCL10 when paired with VAP-1 improves the ability of each factor to diagnose significant fibrosis suggests that these two biomarkers might be incorporated into new clinically testable panels. Greater numbers would also probably show that the combination of VAP-1 and alpha-2 macroglobulin can do the same. Further work should be performed to confirm the mechanism and the link.

Morphometric analysis by CPA of liver fibrosis has shown that the same markers can be linked to quantitatively measured fibrosis. CPA is an emerging technique with the potential to remove inter and intra operator variance although given the need for additional steps in staining and imaging morphometric analysis is likely to remain primarily a research tool.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUDING REMARKS

Liver disease is on the rise and there is an increasing need to be able to accurately assess patients with regard to their fibrosis stage and prognosis so that intervention can be planned in those most likely to respond and with the greatest need. Traditionally liver disease is assessed clinically with the aid of investigations such as liver function blood tests and ultrasound. Accurate assessment of liver fibrosis however has been more difficult and has relied on liver biopsy. Liver biopsy is a flawed technique due to intra and inter-operator variability but it remains the gold standard for assessing fibrosis. It is not without complications and patients will experience pain, as well as being subjected to a small but definite morbidity and mortality risk. This has prompted the search for alternative and potentially better markers of liver fibrosis that are non-invasive.

In this thesis I have summarised the current state of play using non-invasive tests to assess liver fibrosis and suggested new markers that may improve the diagnostic accuracy and clinical utility of current tests.

In chapter 2 we detailed how Fibroscan can be used in a busy liver unit to exclude significant fibrosis. Whilst this is not a new finding, the fact that our study was done in a real clinical setting rather than as part of a research protocol makes it directly applicable to the real world clinic. Our study emphasised the importance of adhering to set criteria that validate the reading and of excluding readings that fall outside these criteria. We showed that if these criteria are not adhered to then the test becomes unreliable and un-interpretable. We have shown that it might be possible in the future to introduce local cut-offs for the

diagnosis of significant fibrosis, although this will complicate comparisons with other departments. In a busy liver unit we have also shown that Fibroscan is an easy skill to learn and one we suggest should be part of a trainees Hepatology training as well as being offered to specialist nurses. This will aid in the efficiency of liver clinics in the future.

In chapter 3 we used systematic review and meta-analysis to show how well non-invasive markers of liver fibrosis predict clinical outcomes and prognosis of chronic liver disease. It is important to note the difference between prediction and prognosis here. Predictive biomarkers identify subpopulations of patients who are most likely to respond to a given therapy. With predictive biomarkers it should be possible to select the therapy with the highest likelihood of efficacy to the individual patient. A prognostic biomarker, provides information on the likely course of the disease in an individual. The main finding of the work is that APRI and Fib-4 can be used to predict death in patients with viral liver disease. This work was however limited by significant study heterogeneity and the use of different cut-offs of the markers involved. This is a field that could be improved by more, large collaborative studies with the implementation of internationalised standards.

In Chapter 4, I report the results of a prospective observational study to analyse a large panel of established and new serum biomarkers to predict fibrosis on simultaneously collected liver biopsy. We showed that commercially available tests such as ELF and Fibrotest accurately predict liver fibrosis as measured by histology. However as detailed above, the gold standard of liver biopsy is flawed because the various stages are not linear in distribution hence the assessment of an alternative analysis of tissue staining using

morphometric analysis. Non-invasive serum markers rely on the fibrotic process to create markers that are shed into circulation that can be detected. A pathologist's staging via Ishak (or other disease specific scores) is not just based on the degree of fibrosis but also the architectural changes. We have shown that VAP-1 and CXCL10 can be used as non-invasive markers of liver fibrosis with AUROC values that approach that of commercially available tests. The study follow up time is too short to make any analysis of the tests ability to predict clinical outcomes but these data will be available as part of the long-term follow up of the NOBLES cohort study.

Also presented in this chapter is the morphometric analysis of liver biopsy samples. I have shown that the technique using standardised settings for each slide yields inaccurate results when compared to Ishak staging and other non-invasive markers. This is improved upon by altering the settings for each slide. The problem with doing this is the potential to introduce bias and also it makes future studies of this technique difficult. As I have shown in chapter 3, any test that has varying cut-offs makes meta-analysis more difficult. It is however a promising new technique that may help pathologists provide individualised information about their patients and therefore improving on their personalised care.

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APPENDICES

6.1 NOBLES Documentation

6.1.1 Data collection sheet Visit 1 and 2

NOvel **B**iomarkers of **L**iv**E**r fibrosi**S** study
NOBLES

Clinical Data Collection sheet Visit 1

Screening Visit
Consent signed?
Date and Time

Patient Initials	DOB
Hospital Number	Sex M/F
Trial ID Number	Exclusion / Inclusion criteria met? Y/N

Liver disease type

Date of diagnosis

Alcohol history

Current Medications

Other PMH

Liver related complications

Observations Temp HR BP Weight Height
BMI
Waist circumference Hip circumference
Examination – any significant findings?
General Appearance
CVS
Resp
Gastro / liver
Neuro
ECG - findings
Blood Tests
Fasted? Y/N
Obtain blood for FBC, PT and INR, U&E, LFTs (AST,ALT, Bili, GGT) AFP and Glucose (1x 4ml purple top, 1x3.5ml blue top, 1x4ml grey top and 1x 4ml yellow top)
Obtain blood for fibromax panel (2x5ml Grey and 2x 5ml Yellow top)
Obtain serum for ELF panel, Fibromax panel, sVAP-1, CK18, serum leptin, IP1-, Il-1B, MCP-1 and fasting insulin as well as storage (4x 6ml Red Top)

Plasma for storage (2x 4ml purple top)

White Cells for storage (1x4ml purple top)

Liver screen bloods to be taken if not already done for HbsAg, HCV Ab, AMA / SMA / Ig's, Ferritin, Caeruloplasmin, A1AT
Fibroscan result kPa IQR: Probe type:

Visit 2 scheduled for:

NOvel **B**iomarkers of **L**iv**E**r fibrosi**S** study
NOBLES

Clinical Data Collection sheet Visit 2

Date and Time

Patient Initials	DOB
Hospital Number	Sex M/F
Trial ID Number	

Current Medications – record any changes

Liver related complications or other significant events since last seen

Observations Temp HR BP Weight Height
BMI
Examination – any significant findings?
General Appearance
CVS
Resp
Gastro / liver
Neuro
ECG - findings
Blood Tests Fasted? Y/N

Obtain blood for FBC, PT and INR, U&E, LFTs (AST,ALT, Bili, GGT) AFP and Glucose (1x 4ml purple top, 1x3.5ml blue top, 1x4ml grey top and 1x 4ml yellow top)

Obtain blood for fibromax panel (2x5ml Grey and 2x 5ml Yellow top)

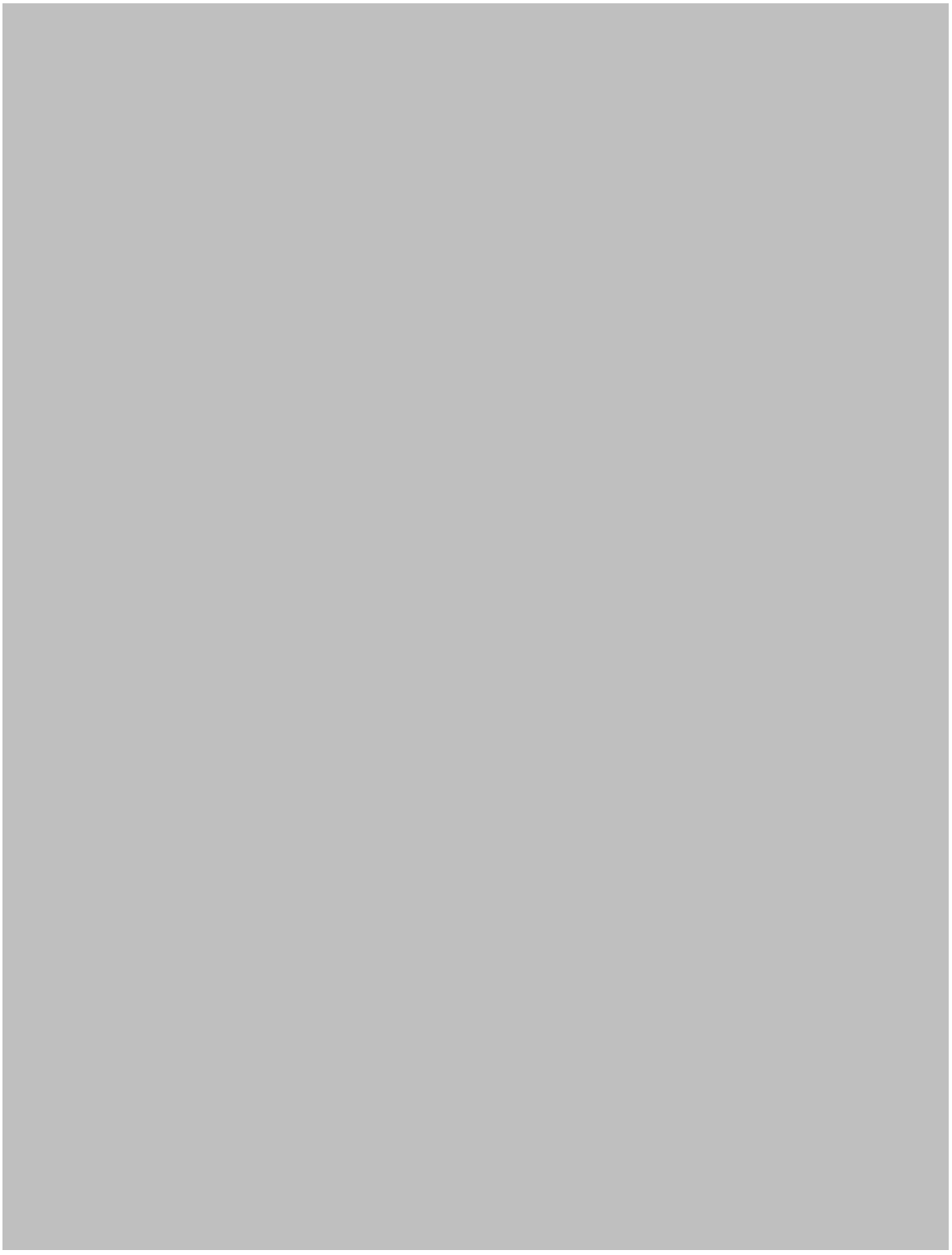
Obtain serum for ELF panel, Fibromax panel, sVAP-1, CK18, serum leptin, IP1-, Il-1B, MCP-1 and fasting insulin as well as storage (4x 6ml Red Top)

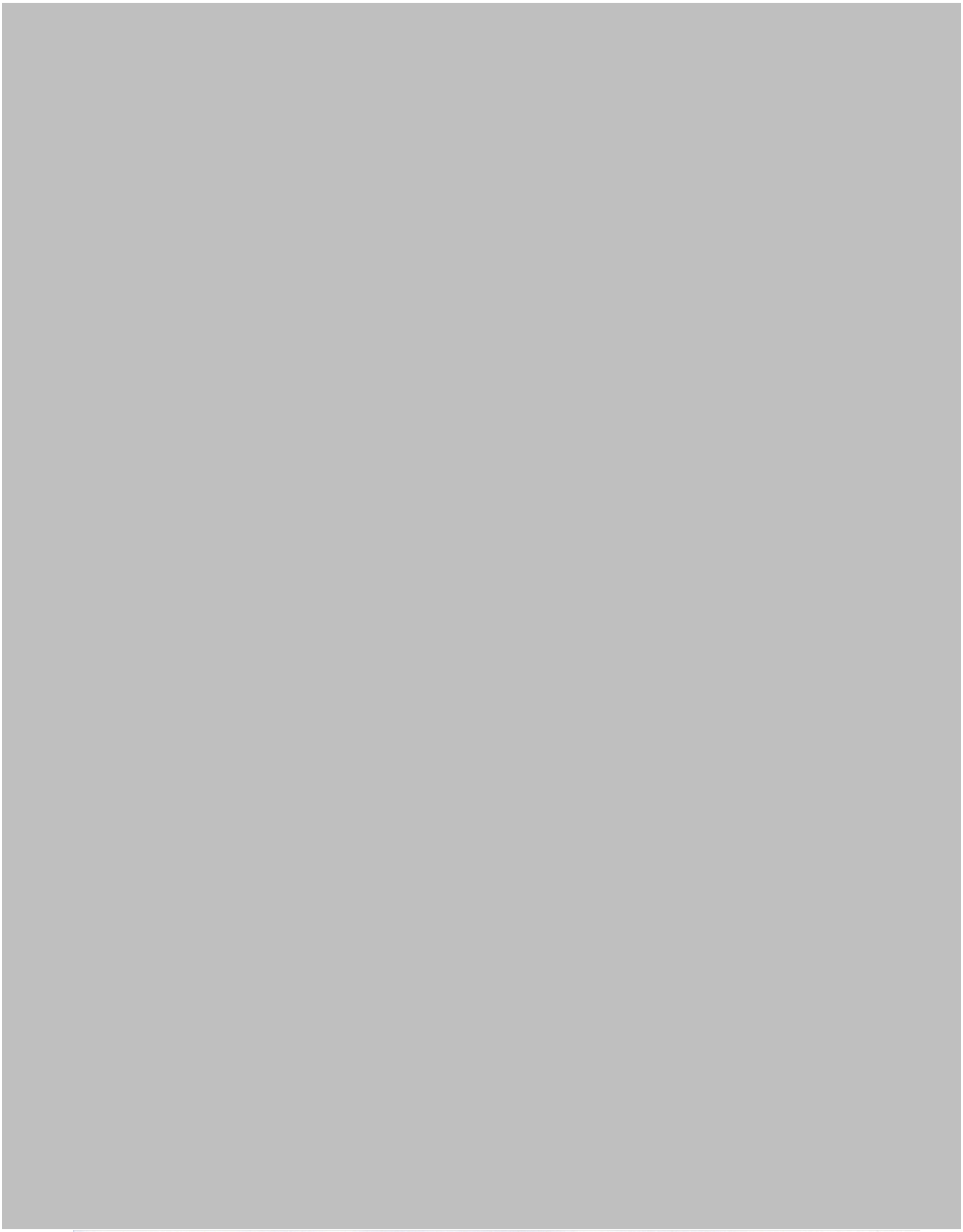
Plasma for storage (2x 4ml purple top)

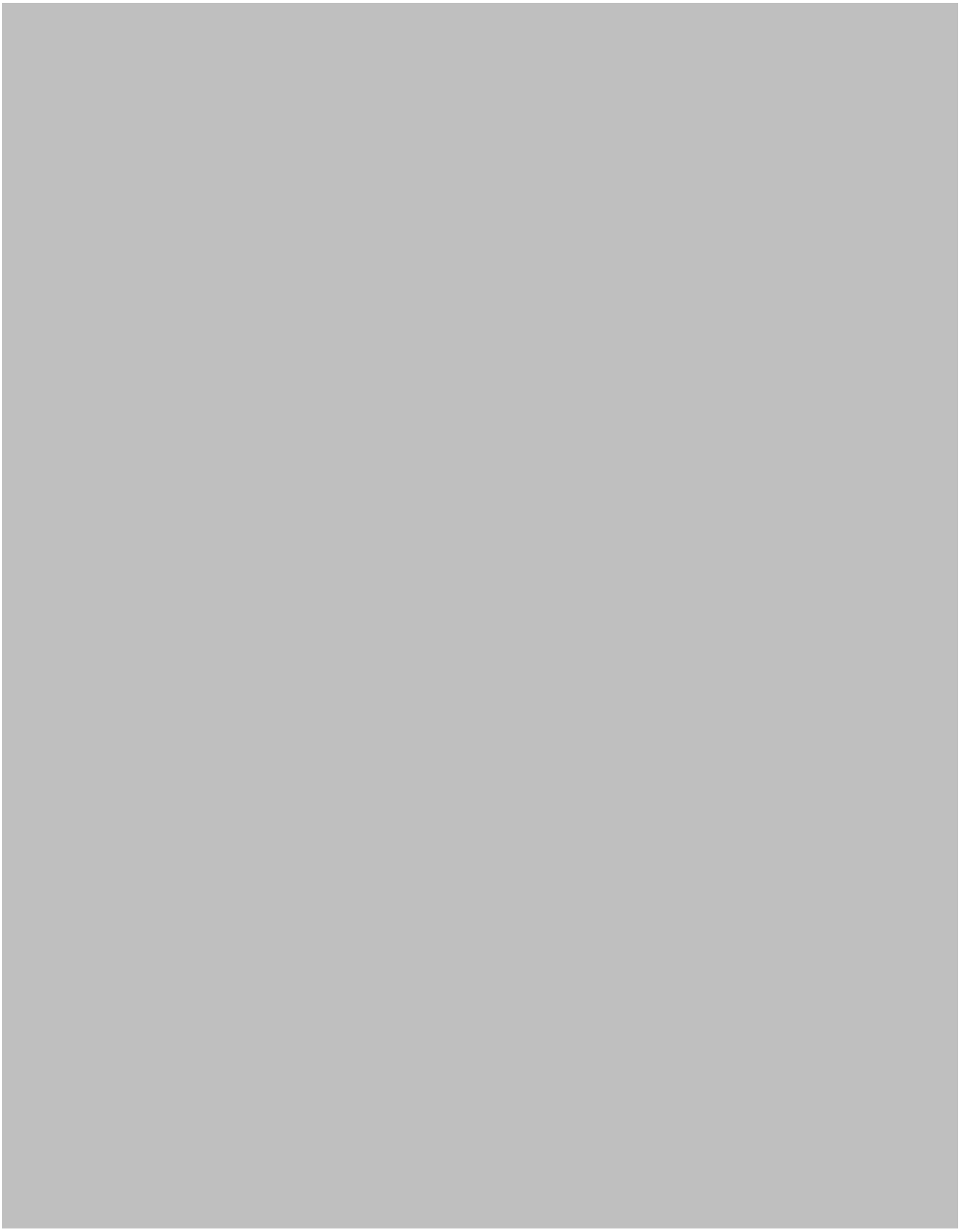
White Cells for storage (1x4ml purple top)

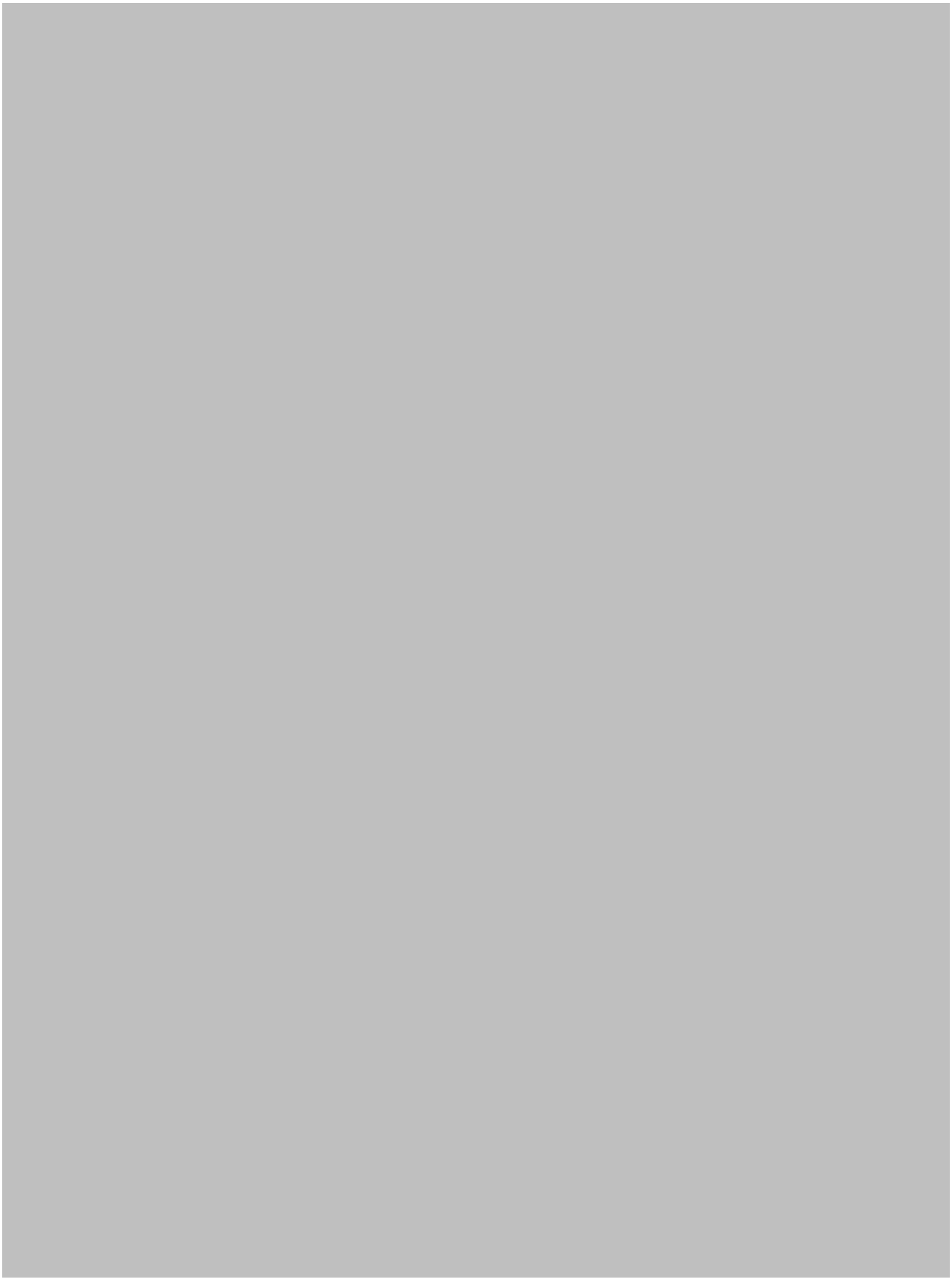
Fibroscan result kPa IQR: Probe type:

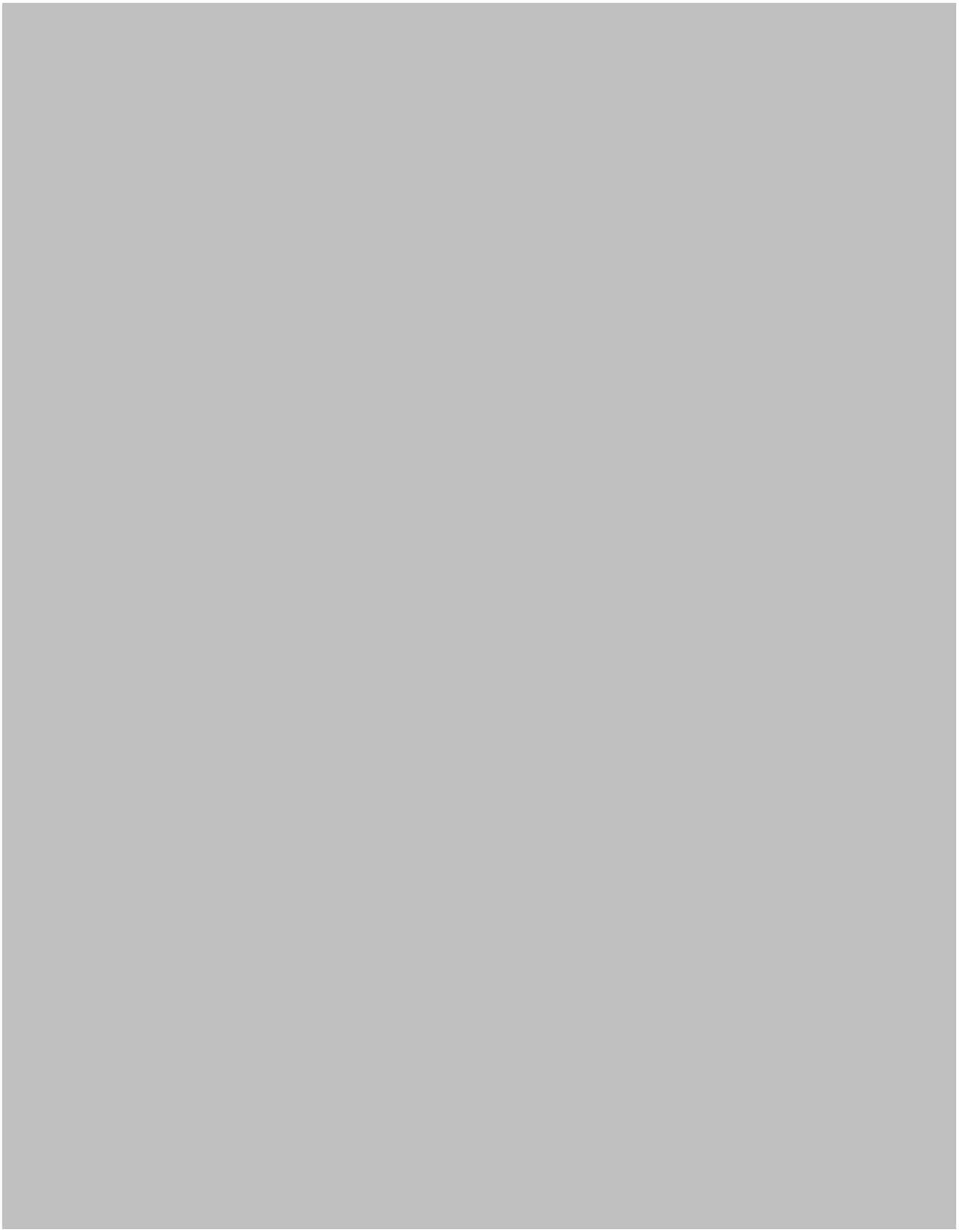
6.1.2 GP and patient information sheets and patient consent form











6.2 Published version of Chapter 2 in Postgraduate Medical Journal entitled "Operator training requirements and diagnostic accuracy of Fibroscan in routine clinical practice"

PGMJ Online First, published on August 7, 2013 as 10.1136/postgradmedj-2012-131640

Operator training requirements and diagnostic accuracy of Fibroscan in routine clinical practice

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ABSTRACT

Background Fibroscan is a quick, non-invasive technique used to measure liver stiffness (kPa), which correlates with fibrosis. To achieve a valid liver stiffness evaluation (LSE) the operator must obtain all the following three criteria: (1) ≥10 successful liver stiffness measurements; (2) IQR/median ratio <0.30 and (3) ≥60% measurement success rate.

Objectives To assess the operator training requirements and the importance of adhering to the LSE validity criteria in routine clinical practice.

Methods We retrospectively analysed the LSE validity rates of 2311 Fibroscans performed (1 August 2008 to 31 July 2011) in our tertiary liver outpatients department at the University Hospital Birmingham, UK. The diagnostic accuracy of Fibroscan was assessed in 153 patients, by comparing LSE (valid and invalid) with the modified Ishak fibrosis stage on liver biopsy.

Results Learning curve analysis highlighted that the greatest improvement in validity of LSE rates occurs in the operator's first 10 Fibroscans, reaching 64.7% validity by the 50th Fibroscan. The correlation between LSE and the fibrosis stage on liver biopsy was superior in patients with a valid LSE (n=97) compared with those with an invalid LSE (n=56) (r_s 0.577 vs 0.259; p=0.022). Area under receiving operating characteristics for significant fibrosis was greater when LSE was valid (0.83 vs 0.66; p=0.048). Using an LSE cut-off of 8 kPa, the negative predictive value of valid LSE was superior to invalid LSE for the detection of significant (84% vs 71%) and advanced fibrosis (100% vs 93%).

Conclusions Fibroscan requires minimal operator training (≥10 observed on patients), and when a valid LSE is obtained, it is an accurate tool for excluding advanced liver fibrosis. To ensure the diagnostic accuracy of Fibroscan it is essential that the recommended LSE validity criteria are adhered to in routine clinical practice.

INTRODUCTION

Chronic liver disease is now the third commonest cause of death in the UK in people under the age of 65 years. Early identification of people with significant liver fibrosis is therefore essential for ensuring the best outcomes from available treatments and preventing premature liver-related deaths. Due to the fact that most patients with chronic liver disease remain asymptomatic until their liver function is compromised, establishing the presence and severity of liver fibrosis remains a clinical challenge. Liver biopsy can accurately confirm the presence of liver fibrosis. However, its invasive nature, the risk of sampling error, interobserver variability and the understandable reluctance of patients to undergo repeat procedures make it an unsatisfactory approach.[1] Consequently, over the last decade, non-invasive tools for identifying liver fibrosis have been developed,[2] with particular focus on the user-friendly technique of Fibroscan (Echosens, Paris, France).

Fibroscan, also called transient elastography, is a non-invasive technique used to provide a rapid measurement of liver stiffness (in kPa) at the bedside. A description of the Fibroscan technique and liver stiffness definitions are summarised in boxes 1 and 2.

To date, large meta-analyses of non-UK studies have shown that the liver stiffness evaluation (LSE) accurately correlates with histological fibrosis (in particular, cirrhosis) in several disease aetiologies.[3–5] The majority of these studies have focused on viral hepatitis (27 studies) and to a lesser extent non-alcoholic and alcoholic steaohepatitis (five studies). According to the manufacturer's criteria,[6] the LSE can be classified as 'valid', 'invalid' or an LSE 'failure' using the parameters that the Fibroscan machine provides (box 2). In research studies, the invalid LSEs are very often excluded from the statistical analyses.[7 8] Therefore, the clinical importance (diagnostic accuracy of Fibroscan) of complying with the LSE validity criteria has never been demonstrated in routine clinical practice. This question has significant implications in the UK and Ireland, as there are currently 134 Fibroscan machines in use in 70 hospitals (information provided by Echosens). Despite this widespread use, there has been a paucity of published Fibroscan data from routine clinical practice in the UK.

The Fibroscan was first introduced at our liver outpatient department at the Queen Elizabeth University Hospital Birmingham (UK) in 2008. Our liver and transplant unit is a tertiary referral centre for populations with varied racial and socioeconomic background in the Midlands and West of England, Wales and Northern Ireland. Since 2008, there has been a gradual increase in the number of LSEs performed per month (2008–2009, 54/month; 2010–2011, 78/month). Between 2008 and 2011, 2311 LSEs were performed as part of the clinical assessment of patients attending the liver outpatients department. Prior to using the Fibroscan machine in clinical practice, the manufacturer (Echosens) provides a small group (2–3 trainees) 4-h training session. The following are discussed: indications for LSE; relevant anatomical landmarks; instructions on how to use the probe; and how to interpret the LSE. Each trainee performed three supervised LSEs on healthy volunteers in the initial training, prior to use on patients with suspected liver disease. There are currently no local, national or international guidelines on how experienced an operator needs to be to achieve consistent and valid LSE readings on patients in the clinic setting.

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Box 1 General information on Fibroscan (transient elastography)

What is a Fibroscan?
- Painless, quick (5–10 min), and non-invasive ultrasound technique, manufactured by Echosens (Paris, France)
- Non-invasive measurement of liver stiffness, which in turn positively correlates with the degree of fibrosis.[3–5]

How does a Fibroscan work?
- It uses a modified ultrasound probe to measure the velocity of an elastic shear wave created by a vibratory source
- The velocity of transmission of the shear wave through the liver is affected by the liver stiffness (ie, the stiffer the tissue, the faster the shear wave propagates).

How is this performed?
- The ultrasound probe is pressed against the skin (intercostal space) overlying the liver with the patient lying supine
- The probe generates a vibration and then measures the velocity of the resultant shear wave as it propagates through the liver.
- The aim is to obtain 10 successful measurements
- The machine then calculates the median value and the IQR of all the successful measurements (in kilopascals, kPa).

The objectives of the current study are to: (1) use statistical modelling to evaluate how many Fibroscans an operator needs to have performed on patients to achieve consistent and valid LSE readings and (2) assess whether obtaining a valid LSE (vs an invalid LSE) affects the diagnostic accuracy of the Fibroscan in routine clinical practice.

METHODS

Study population

All adult patients with suspected chronic liver disease who underwent a Fibroscan as part of their clinical assessment in the 3 years between 1 August 2008 and 31 July 2011 were included in the study to assess the operator training requirements (1st objective). For comparison of the results of Fibroscan with liver biopsy we included those from this group who had a liver biopsy within 12 months of their Fibroscan examination (objective 2). The decision to perform a Fibroscan and to refer for a liver biopsy was made by the specialist hepatologist in clinic (consultant or specialist registrar). Patients with suspected chronic liver disease of any aetiology were included.

Box 2 Liver Stiffness Evaluation (LSE) and definitions

What is the LSE?
- LSE=the median of the successful stiffness measurements (target ≥10)
- The LSE ranges from 2.5 (lowest stiffness) to 75 kPa (highest stiffness)

What is an LSE failure?
- When no stiffness measurements are obtained with 10 attempts

How is the success rate calculated?
- Success rate=number of successful measurements/total number attempted (expressed as %)

What are the manufacturer's LSE validity criteria?
- A 'valid' LSE is classified as obtaining all three of the following[6]:
  - ≥10 successful measurements
  - success rate ≥60%
  - IQR/median ratio <0.30
- An 'invalid' LSE is when one or more of the criteria are not fulfilled

Liver Stiffness Evaluation

Between the study dates, either a consultant hepatologist or a specialist trainee registrar performed the Fibroscan during the outpatient clinic visit. In our unit, all operators underwent a certified training session with an Echosens consultant prior to use in the clinical setting.

All Fibroscans were performed using either the M-probe (3.5 Hz frequency) or XL-probe (2.5 Hz frequency) with the Fibroscan 502 machine (Echosens, France). The manufacturer recommends that the XL-probe should be used in patients with a skin-liver capsule distance >2.5 cm (measured by sonographic imaging). Due to the time constraints in liver clinic, operators were advised to use the XL-probe in patients with a measured Body Mass Index (BMI) >30 kg/m^2.[9] In May 2011, our unit began using the Fibroscan 502 Touch (Echosens, France), which has a built-in automated indicator that recommends the probe best suited to the patient's morphology. In accordance with manufacturer's guidance, all Fibroscans are performed in our clinics with the patient lying in the dorsal decubitus position with the right arm extended. The tip of the ultrasound probe (covered with gel) is placed on the skin in an intercostal space overlying the right lobe of the liver. A time-motion ultrasound image allows the operator to locate a portion of liver at least 6 cm thick and free of large vascular structures or ribs. The median and IQR value of successful liver stiffness measurements (target ≥10) is calculated by the machine and recorded as the LSE. Each LSE was classified as 'valid' or 'invalid' based on the manufacturer's validity criteria[6] (box 2).

Data collection

Data were retrospectively obtained from all three Fibroscan machines in our unit to form a database of the study cohort for assessment of operator training requirements. The Fibroscan parameters that were recorded included: patient identification number, date of Fibroscan, operator, probe, number of successful measurements, success rate and median value (IQR) of successful measurement (known as LSE).

Histopathology reports were then reviewed to identify those patients who had an ultrasound-guided liver biopsy within 12 months of the Fibroscan examination to assess the diagnostic accuracy of the Fibroscan. Demographics, anthropometric measurements (weight, height, BMI), liver enzymes and liver disease aetiology at the time of fibroscan examination were obtained for these cases. The definitive disease aetiology was determined by a combination of the clinical and histological findings and was categorised into fatty liver disease (non-alcoholic or alcoholic), viral hepatitis (hepatitis B, C), autoimmune (autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis), post-transplant and other, for purposes of statistical analysis.

Liver biopsy

Fibrosis staging was used to assess the accuracy of fibroscan for the diagnosis of significant and advanced fibrosis. In our centre, liver biopsies are routinely reported using the appropriate disease-specific liver fibrosis staging (ie, Ishak for hepatitis C;

Kleiner for non-alcoholic fatty liver disease). For purposes of this study, however, each biopsy was reassessed independently by two liver pathologists (NM and RB or NM and SGH) without knowledge of LSE results or other clinical data. In cases of disagreement, a consensus was reached by a joint review. To take account of the diverse aetiologies of liver disease, liver fibrosis was staged using a modified version of the Ishak scoring system,[10] as previously described by Rosenberg *et al*[11] (see online supplementary table S1). Significant fibrosis was defined as a modified Ishak score >2 and advanced fibrosis as a modified Ishak score of 5 or 6. The length of biopsy specimens and the number of portal tracts sampled were recorded as measures of biopsy quality. Biopsies specimens that were deemed not adequate by the pathologists for fibrosis staging were excluded from the analysis.

Statistical analysis

The demographics and characteristics of patients were summarised according to the validity criteria of the LSE (*as defined above*). Continuous variables were compared with independent sample t tests and Mann–Whitney tests (as applicable), and categorical variables were compared with Fisher's exact test.

Operator experience

Binary logistic regression was used to consider the effect of the number of Fibroscan examinations performed on the likelihood of a valid LSE reading. Prior to the analysis, the scan number was $\log_{10}$ transformed, in order that the model was based on the shape of curve generally observed in a learning curve analysis. The results of the analysis were only reported for the first 100 Fibroscan examinations, as some operators had performed fewer than 25 scans. This was in order to maximise their usefulness, while minimising the amount of extrapolation required. However, all the data (n=2311) was used in the production of the statistical model.

Diagnostic accuracy of Fibroscan

The strength of the relationship between the LSE and the modified Ishak score was analysed using Spearman's rho correlation coefficients. Separate coefficients were produced for those measurements where each of the three LSE validity criteria were met (ie, 'valid' LSE), and those where the criteria were contravened (ie, 'invalid' LSE). The coefficients were then compared to test whether non-compliance with the LSE validity criteria is detrimental to the ability of Fibroscan to predict the histological severity of liver fibrosis. The modified Ishak score was then converted into two binary outcomes indicating the presence of significant fibrosis (Ishak 3–6) and of advanced fibrosis/cirrhosis (Ishak 5 or 6). Receiver operating characteristic (ROC) curves were produced to test the accuracy of LSEs in the prediction of significant and advanced fibrosis. Separate ROC curves were produced for LSEs that were deemed 'valid' by each of the validity criteria, and those that were 'not valid', with comparisons made between the resulting areas under the ROC curves (AUROC). A LSE cut-off value of 8 kPa was used to determine the presence of significant fibrosis, above which further investigation is deemed appropriate.[12]

Analyses were performed using IBM SPSS V.19 and Microsoft Excel, with p values less than 0.05 deemed to be indicative of significance.

RESULTS

Effect of operator experience on obtaining a valid LSE (objective 1).

Patients

In the 3-year study period, 2311 LSEs were performed and included in the assessment of operator experience. Of these, 127 (5.5%) were LSE failures, 625 (27.0%) were invalid LSEs and 1559 (67.5%) were valid LSEs (figure 1).

Operator experience

Totally, nine consultants and eight specialist training registrars performed over 25 LSEs each, while a further 29 operators performed less than 25 LSEs each. The most experienced operator performed 670 LSEs, whereas the least experienced performed one in clinical practice (excluding the three performed on healthy volunteers during the initial training day).

Analysis

Binary logistic regression model (figure 2) was used to consider the effect of the number of LSEs performed on the likelihood of a valid LSE, as determined by obtaining the manufacturer's validity criteria. The model shows that a 10-fold increase in the number of LSEs that an operator has performed significantly improves their odds of obtaining a valid LSE (OR 1.57, 95% CI 1.39 to 1.78; p<0.001). Figure 2 shows that only 46% of the initial clinical LSEs performed by an operator were valid, whereas the validity rate rises to 57% by 10 LSEs. After 10 LSEs, the rate at which the operator achieves a valid LSE slows, reaching 64.7% by 50 LSEs and 67.7% by 100 LSEs. In order to obtain a valid LSE, 80% of the time the model forecasts that approximately 2500 LSE would be required.

Importance of the LSE validity criteria for the diagnostic accuracy of Fibroscan (Objective 2).

Patients

In the 3-year study period, 153 (6.6%) patients had a LSE (valid or invalid) that could be compared with liver biopsy (table 1). Of these, 56 (36.6%) patients had an invalid LSE; of which 21 patients (37.5%) had <10 successful measurements, 36 (64.3%) had IQR/median ratio >0.30, and 33 (58.9%) had a success rate <60% (figure 3). The mean age of this group (valid and invalid LSEs) was 48.4 (SE 1.1) years, 68.6% were male, and the mean BMI was 28.4 kg/m^2 (95% CI 27.3 to 29.5). The

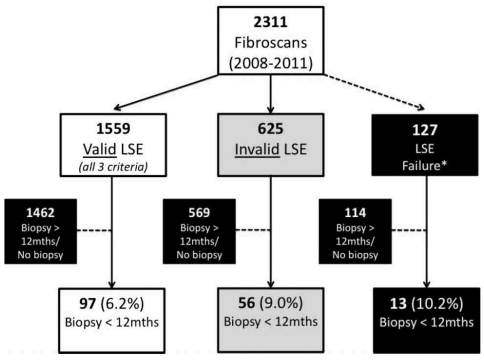

Figure 1 Flow diagram of the entire study; 2311 were included in the operator experience analysis. Of these, 153 patients were selected after exclusion (*black shading*) of patients in which the operator failed to get a single liver stiffness evaluation (LSE) reading (*defined as LSE failure) and/or when biopsy wasn't performed within 12 months of LSE.

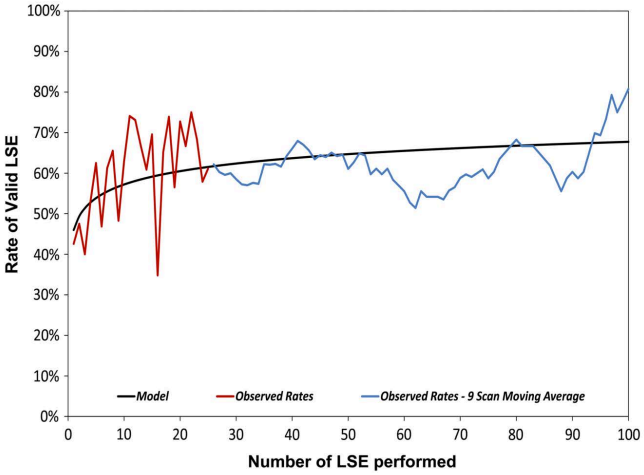

Figure 2 Statistical model (learning curve) to highlight the number of liver stiffness evaluation (LSE) that need to be performed by an operator to achieve a consistent valid LSE. The black line represents the model produced from the binary logistic regression analysis. For scans 1–25, the rates of validity across all operators are plotted at each scan number (the red line). Since the number of operators drops off sharply (n=46 to 17 operators) after this point, the subsequent scans are summarised as a nine-point moving average, in order to isolate the trend from variability in the data. The model seems to be a reasonable fit to the observed data, suggesting that it is a valid summary of the general trend.

disease aetiologies (confirmed on biopsy) were fatty liver disease in 37.9% (n=58), viral hepatitis in 32.0% (n=49), autoimmune in 8.5% (n=13), post-transplant in 9.8% (n=15) and miscellaneous/other in 11.8% (n=18).

Liver histology and LSE

The median time difference between LSE and liver biopsy was 70 days (IQR 22.0–127.0). The mean number of portal tracts and length of biopsy was 14.9 (95% CI 13.9 to 16.1) and 15.7 (95% CI 14.9 to 16.5) mm, respectively. The liver pathologists deemed all 153 liver biopsies adequate for fibrosis staging. Seventy patients (45.7%) had significant fibrosis (Ishak stage 3–6), of which 25 had advanced fibrosis (Ishak 5–6). Seventy-eight (51.0%) of the LSEs were performed by consultant hepatologists, with the remainder by specialist registrars in training. One hundred and six (69.2%) of the LSEs were performed using the M-probe versus 47 (30.8%) with the XL-probe. Overall, the median LSE for the population of readings was 10.2 kPa (IQR 6.8–17.1).

Analysis

LSEs were significantly higher in patients with an invalid scan compared with those with a valid scan (14.1 vs 9.4 kPa; p=0.011). This was most pronounced in patients without fibrosis on biopsy (12.9 vs 5.6 kPa; p=0.008). There was no significant difference in age, sex, disease type, BMI, aspartate transaminase and histological parameters between patients with a valid LSE and those with an invalid LSE (table 1). The correlation between LSE and modified Ishak fibrosis stage was significantly superior in patients with a valid LSE compared to those with an invalid LSE (r_s 0.577 vs 0.259; p=0.022) (figure 4).

The accuracy of LSE (valid vs invalid) in predicting significant and advanced fibrosis was analysed using AUROC. The AUROC for significant fibrosis (Ishak 3–6) was significantly greater with a valid LSE than an invalid LSE (0.83 vs 0.66; p=0.048). There was no significant difference in the AUROC for advanced fibrosis (Ishak 5–6) between a valid LSE and an invalid LSE (0.87 vs 0.76; p=0.361).

The published[12 13] LSE cut-off of 8 kPa was used to determine the sensitivity, specificity, negative predictive value (NPV) and positive predictive value for the presence of significant (Ishak 3–6) and advanced fibrosis (Ishak 5–6) (table 2). A valid LSE produced a sensitivity of 86% (95% CI 71% to 95%) and specificity of 58% (95% CI 44% to 71%), whereas an invalid LSE resulted in a sensitivity of 84% (95% CI 64% to 95%) and a specificity of 42% (95% CI 22% to 63%). Subsequently, the NPV for the presence of significant fibrosis was 84% for a valid LSE compared with 71% for an invalid LSE. Furthermore, the NPV for presence of advanced fibrosis was 100% for a valid LSE versus 93% for an invalid LSE (table 2).

DISCUSSION

Our large retrospective, single-centre study (n=2311) highlights that Fibroscan requires minimal operator training (≥10 observed on patients). The greatest improvement in ability to achieve a valid LSE occurs in the operator's first 10 scans (46–57%), and thereafter the validity rate progressively increases, albeit very slowly. Second, our subgroup analysis of patients who underwent a liver biopsy (n=153) highlights that importance of adhering to the manufacturer's recommended LSE validity criteria. Obtaining a valid LSE (vs invalid LSE) resulted in a significantly greater correlation with liver fibrosis stage and greatly enhanced the accuracy of a negative LSE in ruling out significant (using LSE cut-off >8 kPa, NPV 84%), and advanced liver fibrosis (NPV 100%).

Clinical findings and implications compared to previous studies

It is important to understand what defines adequate Fibroscan training prior to widespread incorporation into UK clinical practice (including the potential for community-based assessment[13]). Previous hospital-based studies have reported contrasting degrees of operator experience that are required to achieve consistent and valid LSE readings (range 20 to >500 LSEs required [6 13–15]). Our statistical model highlights that the initial supervised period should incorporate a minimum of 10 Fibroscans on patients with suspected liver disease, to ensure that the trainee has the expected improvement in validity rate. Thereafter, 50 Fibroscans should achieve a stable degree of consistency in valid LSE rates. Furthermore, LSE validity rates were not affected by the grade of

Table 1 Demographics and characteristics of patients who underwent Fibroscan and liver biopsy

	Validity of LSE		
	Valid (n=97)	Not valid (n=56)	p Value
Characteristics			
Age (years)†	47.4 (1.4)	50.1 (1.5)	0.232
Gender			0.718
Male	68 (70.1%)	37 (66.1%)	
Female	29 (29.9%)	19 (33.9%)	
Disease type			0.678
Fatty liver disease (NAFLD/ALD)	39 (40.2%)	19 (33.9%)	
Viral (HBV/HCV)	28 (28.9%)	21 (37.5%)	
Autoimmune (AIH/PSC/PBC)	7 (7.2%)	6 (10.7%)	
Post-transplant	10 (10.3%)	5 (8.9%)	
Other	13 (13.4%)	5 (8.9%)	
BMI (kg/m^2)	27.9 (26.7 to 29.2)	29.2 (27.3 to 31.2)	0.263
AST (U/L)	49.9 (44.3 to 56.3)	48.4 (40.2 to 58.1)	0.762
Liver biopsy			
Time difference between biopsy and Fibroscan (days)‡	70.0 (23.5 to 122.5)	69.0 (12.8 to 195.0)	0.953
Portal tracts (n)	15.1 (13.8 to 16.6)	14.6 (12.7 to 16.7)	0.630
Length of biopsy (mm)	15.4 (14.5 to 16.5)	16.1 (14.7 to 17.6)	0.477
Modified Ishak Stage of Fibrosis (0–6)			0.387
0	14 (14.4%)	10 (17.9%)	
1	24 (24.7%)	13 (23.2%)	
2	17 (17.5%)	5 (8.9%)	
3	18 (18.6%)	9 (16.1%)	
4	8 (8.2%)	10 (17.9%)	
5	11 (11.3%)	4 (7.1%)	
6	5 (5.2%)		
Fibroscan			
Operator			0.738
Consultant	49 (50.5%)	30 (53.6%)	
Specialist registrar	48 (49.5%)	26 (46.4%)	
Probe			0.856
M-probe	68 (70.1%)	38 (67.9%)	
XL-probe	29 (29.9%)	18 (32.1%)	
LSE (kPa)‡	9.4 (6.6 to 14.5)	14.1 (7.3 to 26.1)	0.011§,*
LSE per modified Ishak Stage (kPa)‡			
0	5.6 (4.7 to 6.8)	12.9 (6.8 to 17.1)	0.008§,*
1–2	8.6 (6.5 to 10.9)	8.5 (6.1 to 18.8)	1.000§
3–4	11.4 (8.7 to 20.0)	16.0 (8.9 to 18.6)	1.000§
5–6	17.3 (12.1 to 26.0)	48.9 (11.9 to 68.2)	0.612§

Continuous data displayed as: Geometric Mean (95% CI) Categorical data displayed as: N (%).
*Significant=p<0.05.
†Data displayed as: Mean (SE).
‡Data displayed as: Median (Quartiles).
§p Values Bonferroni-Adjusted for 4 comparisons.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; AST, aspartate transaminase; BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; LSE, liver stiffness evaluation; NAFLD, nonalcoholic fatty liver disease; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

the doctor (consultant vs specialist registrar, p=0.738). This is in keeping with previous studies that recommend that a novice, of any medical professional status, can be trained to use Fibroscan.[13 15]

By contrast with previous research studies,[7 8 16] our study highlights for the first time in UK clinical practice that complying with the recommended LSE validity criteria (box 2) provides better diagnostic accuracy than invalid LSE. Our data suggests that failure to meet the LSE validity criteria increases the risk of overinterpreting an LSE >8 kPA and incorrectly labelling a patient as having significant fibrosis, in those without fibrosis (figure 4). Furthermore, after obtaining an invalid LSE <8 kPa the clinician runs the risk of falsely reassuring 7% patients who have underlying advanced fibrosis (Ishak 5–6). By contrast, when a valid LSE is performed, clinicians (consultant or registrar level) can exclude significant fibrosis, and to a greater extent advanced fibrosis, with a high degree of confidence. In our unit, using the cut-off of 8 kPa with a valid LSE, we could reliably exclude significant and advanced fibrosis (sensitivities Ishak >2=86%; Ishak 5–6=100% and NPVs Ishak >2=84%; Ishak 5–6=100%) as effectively as data reported in large prospective studies,[14 17 18] nurse-based studies[13] and recent meta-analyses.[3–5] In order to reduce the number of false positive LSEs in our centre, while ultimately maintaining the ability to exclude advanced fibrosis (ie, NPV 100%), a cut-off of 10 kPa could be adopted (data not shown). Even by increasing the valid LSE cut-off further, our reported

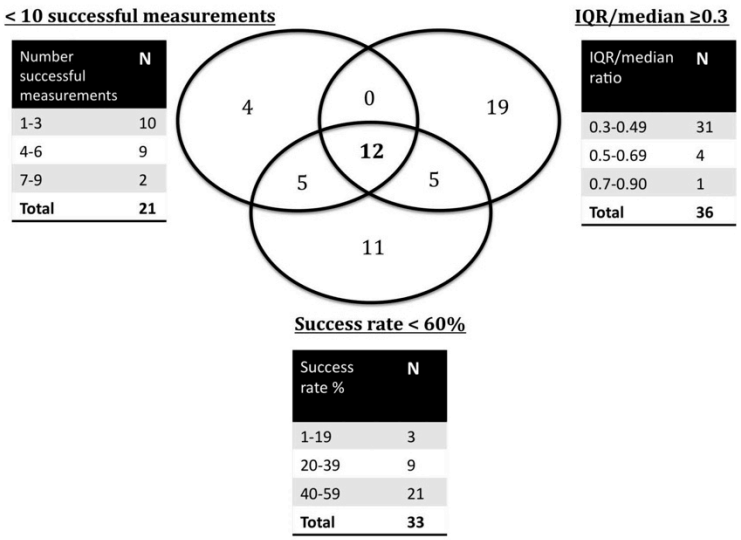

Figure 3 Venn diagram highlighting the reason(s) for an invalid liver stiffness evaluation (LSE) in the biopsied patients only. 56/153 patients had an invalid LSE. Tables highlight the distribution of spread for each of the three reasons that the LSE was classed as invalid.

number of false positives (LSE over estimates) outweigh those recently reported in a large 40 study meta-analysis by Tsochatzis *et al*.[3]

Limitations and strengths of the study:

Only a small percentage of the patients (153/2311; 6.6%) included in the study had a liver biopsy within 12 months, to

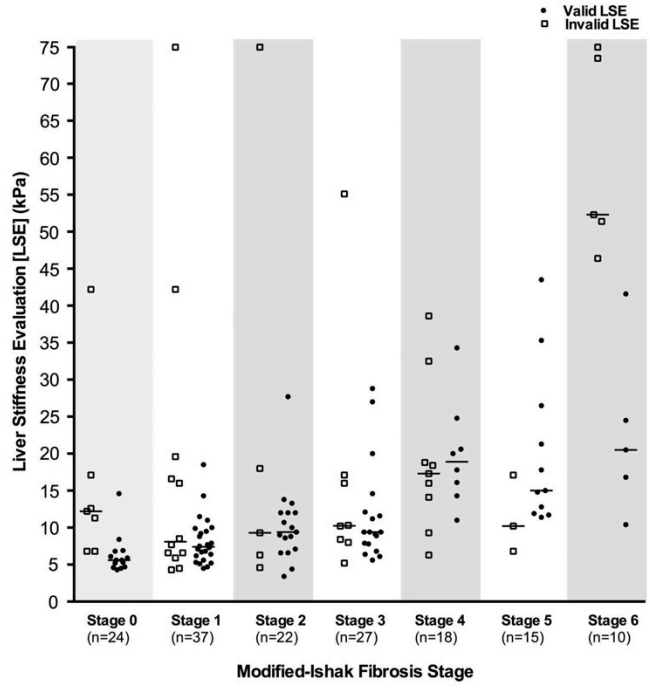

Figure 4 Relationship between the liver stiffness evaluation (LSE) and the modified Ishak stage of liver fibrosis for both valid LSE (*black circle*) and invalid LSE (*white box*). Median LSE values for each modified Ishak stage represented by horizontal bar.

Table 2 Importance of the LSE validity criteria (cut-off > 8 kPa) for diagnostic accuracy of (a) significant and (b) advanced fibrosis

LSE validity criteria	Sensitivity %	Specificity %	PPV %	NPV %
a. Significant fibrosis (Ishak 3–6)				
Invalid LSE	84 (64 to 95)	42 (22 to 63)	60 (42 to 76)	71 (42 to 92)
Valid LSE	86 (71 to 95)	58 (44 to 71)	61 (47 to 73)	84 (69 to 94)
b. Advanced fibrosis (Ishak 5–6)				
Invalid LSE	88 (47 to 100)	32 (18 to 48)	20 (8 to 37)	93 (66 to 100)
Valid LSE	100 (79 to 100)	47 (36 to 58)	27 (16 to 40)	100 (91 to 100)

Sensitivity, specificity and positive/NPVs of LSE for 153 patients.
95% CIs in brackets. LSE validity criteria as per Castera *et al*.[6]
LSE, liver stiffness evaluation; NPV, negative predictive value; PPV, positive predictive value.

enable the diagnostic accuracy of Fibroscan to be determined. This introduces an unavoidable selection-bias that accompanies routine clinical decision-making, and provides a possible explanation for the high false positive rate of LSE in our study. For instance, in the event that the clinician has a low clinical suspicion of advanced liver fibrosis, he/she is more likely to proceed to a liver biopsy in the event of an unexpected high LSE compared with an expected (confirmatory) low LSE. A clinically relevant question would be to investigate how many patients in routine practice avoided liver biopsy as a direct result of the Fibroscan result. After the introduction of Fibroscan in our unit in August 2008, we saw a reduction in the number of outpatient liver biopsies (134 biopsies between 1 February 2008 and 1 July 2008; 89 biopsies between 1 February 2009 and 1 July 2009) (personal communication, Dr D Freshwater). This would imply that the Fibroscan influenced the clinicians' decision making, but to answer this question accurately would require prospective cohort study (ie, using questionnaires).

Due to time constraints it was routine practice in our centre to use the measured BMI (cut-off 30 kg/m^2 [9]) to determine the correct probe to use, rather than measure the skin to liver capsule distance (as used in previous research studies). This may have resulted in the inappropriate use of the M-probe in cases of >2.5 cm subcutaneous adipose tissue (despite a BMI <30 kg/m^2) and therefore overestimates of LSE, as previously reported with the M-probe.[9] The sample size prior to and following the introduction of Fibroscan 502 Touch, which automatically informs the operator of which size probe to use, is too small to determine the impact of the new model of Fibroscan on the diagnostic accuracy in our centre. As in all studies that use liver biopsy to evaluate the performance of Fibroscan, interobserver agreement and sampling error in fibrosis staging must be considered.[19] In order to minimise these limitations, three liver pathologists (RB, SGH, NM) restaged liver fibrosis and reached a consensus in cases of disagreement (<10% cases). Given that the median time delay between Fibroscan and biopsy was 70 days (IQR 22–127) it is unlikely that progression of fibrosis could have contributed to discordance. Furthermore, time delay between Fibroscan and biopsy was not a predictor of false positives/negatives in our study (data not shown). Due to the sample size of our heterogeneous cohort, the employed LSE cut-offs for significant/advanced fibrosis were generic[5 12 13] and not specific to individual disease aetiology and/or probe use.

Outstanding research questions

Disease-specific and probe-specific LSE cut-offs for advanced fibrosis still require validation in UK clinical practice with prospective study. The methodological challenges of comparing Fibroscan with histological fibrosis in clinical practice are well documented.[19] Future studies should, therefore, focus on investigating the influence of the LSE validity criteria (and modified versions[20]) and the accuracy of Fibroscan in predicting clinical events (ie, liver failure, hepatocellular carcinoma, death, etc).

SUMMARY

This study should inform other UK National Health Service centres that prior to using Fibroscan in clinical practice, novices should be trained to understand the clinical implications of the LSE validity criteria, and should undertake a minimum of 10 observed scans on patients prior to using the Fibroscan independently.

Main messages

- Fibroscan requires minimal operator training (≥10 observed scans on patients) prior to independent clinical use.
- The liver stiffness evaluation (LSE) validity criteria should be adhered to in clinical practice to ensure diagnostic accuracy.
- A valid LSE is an accurate, non-invasive tool for excluding advanced liver fibrosis

Current research questions

- What is the impact of Fibroscan on the clinical decision-making process? (ie, does it determine the requirement for liver biopsy, the choice of treatment and the decision to discharge from follow-up?)
- How accurate is Fibroscan (and liver stiffness evaluation validity criteria) in predicting clinical events (ie, liver failure, cancer, death)?
- What is the diagnostic accuracy and feasibility of Fibroscan in primary care?

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