UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

SCHOOL OF MEDICINE

Exploring the diagnostic tools for the assessment of liver fibrosis using biofluids

By

Indra Neil Guha

Thesis for the Doctor of Philosophy

August 2007

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

SCHOOL OF MEDICINE

Doctor of Philosophy

Exploring the diagnostic tools for the assessment of liver fibrosis using biofluids

By Indra Neil Guha

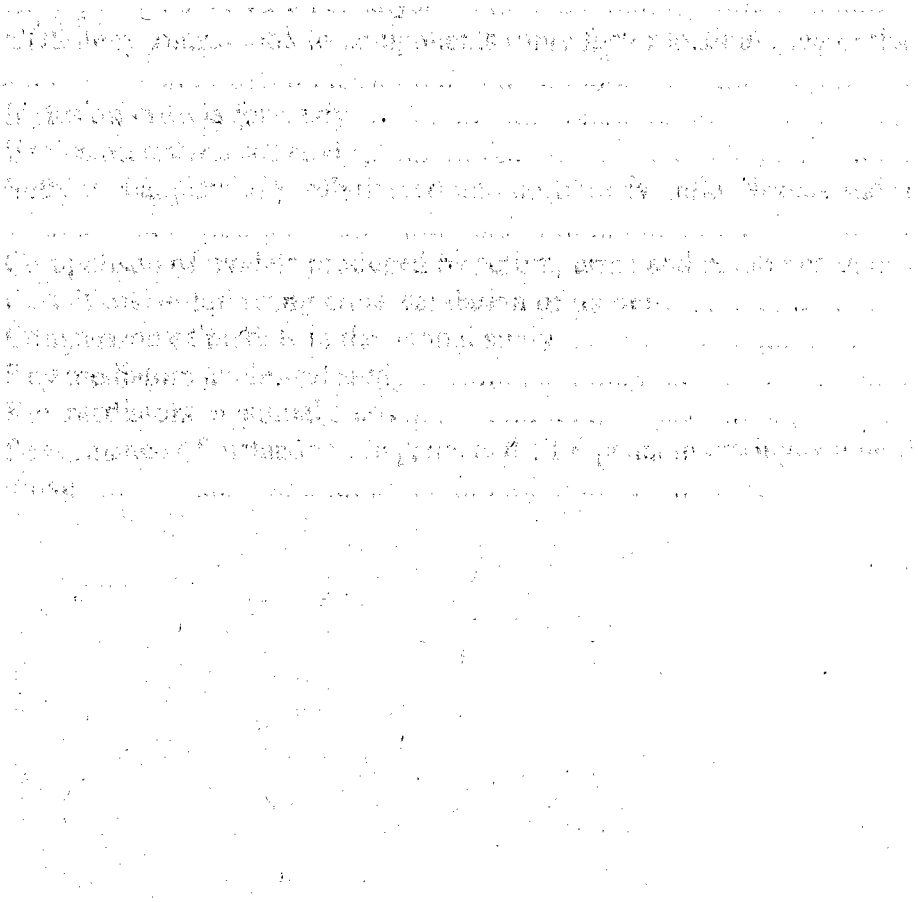
Liver fibrosis is a common pathway of injury following chronic insult to the liver. The evolution of liver fibrosis to cirrhosis has a wide number of clinical implications including bleeding, infection, hepatocellular carcinoma and death. The reference standard for the diagnosis of liver fibrosis is currently histological assessment of tissue obtained by liver biopsy. Whilst this provides valuable information it has limitations that include: being an invasive procedure, sampling error, observer variability and the use of categorical scoring systems.

This thesis focussed on non-invasive markers of liver fibrosis measured in biofluids and had three broad aims. Firstly, the evaluation of non-invasive markers in the literature in two major causes of liver disease, chronic hepatitis C (CHC) and non-alcoholic fatty liver disease (NAFLD). Secondly, the exploration of how non-invasive markers could be used in clinical practice. Thirdly, the investigation of a novel technology to improve the diagnostic accuracy of non-invasive markers.

The systematic reviews highlighted the breadth of serum markers that are potentially available for the diagnosis of liver fibrosis and how the field has evolved from the use of single markers in NAFLD to panel markers in CHC. Simple markers, identified in the systematic review, were combined with a panel marker test to assess if diagnostic performance could be improved. A clinical utility model was developed to show how diagnostic tests can be used in clinical practice. Using this model 86 % of liver biopsies could be avoided using a novel combination of panel markers for the diagnosis of severe fibrosis in NAFLD. The final part of the thesis explored how a technology platform, metabonomics, could aid the development of diagnostic markers. In this part of the study a number of signals were found to alter in early fibrosis, including mediators related to glucose metabolism. The potential to use metabonomics as a diagnostic is limited by practical considerations but it may have a role in highlighting critical pathways of disease.

Current non-invasive biomarkers have a role in the assessment of liver fibrosis and continuing to improve the accuracy and spectrum of diagnosis of non-invasive markers will increase the application of these tests in routine clinical practice.

Contents



List of tables

List of figures

Author's declaration

The work presented in this thesis was performed entirely during candidature for the degree of Doctor of Philosophy form the University of Southampton under the supervision of Professor Rosenberg, Professor Roderick and Professor Iredale. It is my own original work. Significant contributions by collaborators have been acknowledged in each chapter. All major publications resulting from this work are shown in Appendix 10. The material in this thesis has not been submitted, either in part or whole, for a degree or other qualification at another academic institution.

Acknowledgements

I would like to thank the following people, departments and organisations:

My supervisors William Rosenberg, Paul Roderick and John Iredale. Firstly, for giving me the opportunity to pursue this research. Secondly, they have given me support, guidance, teaching and constant encouragement. Their mentorship and belief in me has been greatly appreciated.

All the staff at the Public Health Medical Sciences and Statistics who have accommodated and welcomed me into the department.

I performed the systematic reviews with Julie Parkes. She was an invaluable colleague and friend during my thesis.

Professor Chris Day and Dr Guruprasad Aithal were collaborators for the NAFLD study. I am grateful for their helpful advice during this part of the study.

Pfizer provided the facilities to undertake the metabonomics study. In particular I would like to thank Philip Murphy, Geoff Johnston, Olaf Beckonert and Ross Walley.

The Wellcome Trust Clinical Research Facility at Southampton General hospital for providing a supportive environment to undertake clinical research.

The clinicians, patients and nursing staff at Southampton University General Hospital, Royal Bournemouth Hospital, Nottingham University Hospital and Newcastle Upon-Tyne Hospital without whose time and consideration this research would not have been possible.

My examiners Professor Mark Thursz and Professor Paul Little for their time, critique and valuable suggestions for this thesis.

My mother Sipra, my late father Ranjit and sister Anita for inspiration and encouragement. Finally, my wife Josephine for her unconditional support and understanding during the thesis and our first son Daniel.

Rationale for this thesis

Deaths from liver cirrhosis in the UK are increasing; in 2001 the Chief Medical Officer reported an eight fold increase in the death over the last 30 years in men aged 35-44 and a seven fold increase in women aged 35-44 [1]. The burden of chronic liver disease in the UK is high and rising and the three main causes are alcohol, chronic hepatitis C (CHC) and non-alcoholic fatty liver disease (NAFLD). This will lead to higher rates of cirrhosis over the next few decades as a significant proportion of patients with such chronic liver disease will develop liver fibrosis and cirrhosis (eg 20-25 % in CHC and 20-30 % in NAFLD).

Conservative estimates of chronic hepatitis C in the UK suggest at least 250,000 individuals, however because of the difficulties of ascertaining disease estimates in vulnerable groups such as intravenous drug abusers and prisoners the true prevalence may be much higher [2]. The epidemic of obesity is leading to complications of type 2 diabetes, metabolic syndrome and non-alcoholic fatty liver disease. It has been estimated that NAFLD may affect 20-30 % of the general population in western countries[3].

There are a number of reasons why it is important to have a safe and effective diagnostic tool for liver fibrosis. Patients with liver fibrosis represent the group at greatest risk of developing liver associated morbidity and mortality and thus are in greatest need of therapy for the underlying aetiology (eg antiviral treatment for CHC, abstinence for alcohol and weight loss/ possibly glitazones for NAFLD). Secondly, prognostic information for utilisation by patients and clinicians is dependent on assessing disease severity and understanding factors associated with progression. Thirdly, where it is not possible to remove the underlying insult, specific anti-fibrotic therapy in the future may have an important role; the potential target population will need to be identified and the effectiveness of any therapy measured in terms of reducing progression of fibrosis. Finally, liver fibrosis is generally asymptomatic until the final stages of disease. Thus the estimation of the prevalence of significant disease (i.e. fibrosis or cirrhosis) requires diagnostic tests. It may be underestimated currently for example due to under-ascertainment on death certification, social stigma of certain aetiologies of liver disease and reliance on an invasive method of diagnosis. To

plan preventative and therapeutic interventions effectively, the incidence and prevalence of liver disease of different severities (from mild fibrosis to cirrhosis) will need to be established in the population. Additionally, causal factors associated with fibrosis will need to be determined. Such epidemiological studies require simple, robust non-invasive measures.

Diagnostic tests are well established for the underlying liver aetiology, eg blood tests measuring genotype and viral load in chronic hepatitis B and C or iron studies in serum in combination with genotype for haemachromatosis. Critically however, the diagnosis of liver fibrosis is currently reliant on histological assessment of liver tissue. Whilst this has been the reference standard for the general diagnosis of liver disease it has several problems and is imperfect for the assessment of fibrosis. It is invasive and in large studies complications of pain (20%), serious morbidity (0.6%) and mortality (0.01%) are reported [4]. The biopsy is subject to sampling error; studies have shown a 30 % difference in staging of disease when biopsies are taken from the right and left lobe of the liver of the same patient at open laparoscopy [5]. Interpretation of biopsies by pathologists is subject to intra- and inter- observer variability. Furthermore, the scoring systems used have inherent constraints because they are based on ordinal, categorical variables and due to the invasive nature of biopsy there are ethical considerations on the intervals between biopsies.

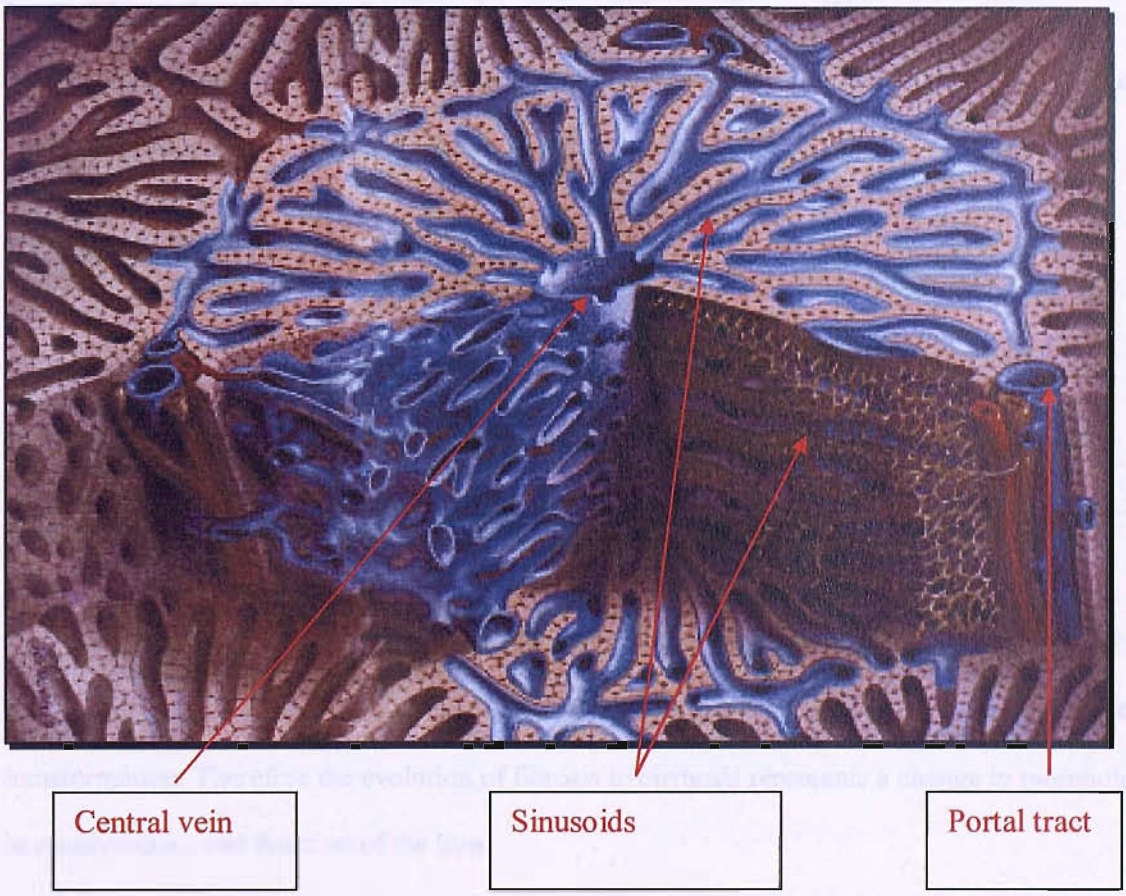
This thesis is divided into three areas. 1) The evaluation of non-invasive markers in the literature in two major causes of liver disease, chronic hepatitis C and NAFLD. 2) The exploration of how non-invasive markers could be used in clinical practice. 3) The investigation of new technologies that may potentially improve the diagnostic accuracy of non-invasive markers.

Chapter 1 : Introduction to liver fibrosis and cirrhosis

1.1 Structure and function of the normal liver

The liver is the largest organ in the body and weighs between 1 to 1.5 kg. It has a dual blood supply; the portal vein brings the majority of blood (70-80 %) from the intestines and spleen and the hepatic artery is a branch of the coeliac axis. The vessels enter the liver through the porta hepatitis before dividing into branches to the right and left lobe. The basic structure of the liver consists of a hepatic lobule comprising a central hepatic vein with peripheral portal tracts (each containing a bile ductule, portal venule and hepatic arteriole). Blood flows from these portal tracts to the central vein via sinsoids.

Figure 1-1: Schematic diagram of lobule with central hepatic vein, sinusoids and peripheral portal tract.



The sinusoids are specialised capillaries and are lined by a fenestrated endothelial barrier. This facilitates exchange between incoming blood and hepatocytes thorough the space of Disse. Resident macrophages of the liver, Kupffer cells, lie in close proximity to the sinusoids and have a role in the clearance of endotoxins, immune complexes and senescent red blood cells. Furthermore, they have a role in the innate immune response and produce pro-inflammatory cytokines. The hepatic stellate cells also have a peri-sinusoidal location. They store vitamin A but on activation transform into alpha- smooth muscle actin-positive cells and have a role in regulation of microvascular tone and the synthesis of extracellular matrix.

The liver has a number of diverse functions. It has a central role in the metabolism of amino-acids (eg transamination), carbohydrates (eg glycolysis and gluconeogenesis), lipids (eg cholesterol production), haemoglobin, bile salts, iron, copper, vitamins, ammonia and drugs. The liver is a major synthetic organ for the production of albumin, serum binding proteins (e.g. haptoglobin and sex hormone binding globulin) and clotting factors. It is an important immunological site (eg cytokine signalling, antigen surveillance and immune tolerance) and has an important excretory function in producing bile.

1.2 Evolution of fibrosis to cirrhosis

Fibrosis is part of the innate wound healing response which occurs in injured tissues. Within the liver, fibrosis is characterised by the deposition of extracellular matrix. Current evidence indicates that net deposition of matrix is the result of a balance between synthesis and degradation and constitutes a dynamic process. The progression of fibrosis to cirrhosis has a number of sequale. Firstly it will distort hepatic architecture and vasculature, secondly it will have a deleterious effect on hepatic function and thirdly it will increase the propensity for neoplastic transformation. Therefore the evolution of fibrosis to cirrhosis represents a change in morphology, haemodynamics and function of the liver.

The progression of liver fibrosis occurs by a number of mechanisms including iterative injury, apoptosis and collapse, inflammation, contractility and angiogenesis. These will be briefly discussed.

Iterative injury

It is self evident that iterative injury is an important and common mechanism by which fibrosis progresses to cirrhosis. This is exemplified by clinical paradigms where the injurious insult is removed. In alcoholic liver disease, abstinence from alcohol not only prevents disease progression but be associated with regression. Similarly, anti viral treatment in hepatitis B and hepatitis C and venesection in haemachromatosis are therapeutic strategies which can prevent the development of cirrhosis.

Apoptosis and collapse

Reduced hepatic cell mass is a key feature of advancing liver disease; apoptosis and collapse both contribute to this.

Toxic injury to hepatocytes can initiate "programmed" cell death, a process known as apoptosis. However, apoptosis may also augment inflammation, by the direct release of cellular contents such as cytokines or by the signals that induce apoptosis also co-stimulating inflammatory cascades [6]. Apoptosis results in the fragmentation of cells into membrane bound bodies called apoptotic bodies. The clearance of apoptotic bodies has classically been viewed as not inducing inflammation but in certain liver models it has been shown to be fibrogenic [7]. Additionally secondary necrosis can supervene in the context of massive apoptosis when clearance mechanisms are overwhelmed. Therefore, apoptosis not only causes a loss of hepatocytes but also contributes to inflammation and fibrosis which in turn, leads to further apoptosis. Finally, the complex interaction of apoptosis and fibrosis is highlighted by the role of apoptosis in the resolution of fibrosis. It has been shown that loss of activated hepatic stellate cells, mediated by apoptosis, occurs in the recovery phase of fibrosis[8]. Moreover gliotoxin, which is a fungal metabolite that induces apoptosis in activated HSCs, ameliorates fibrosis in a carbon tetrachloride rat model[9].

Collapse of architecture results in the approximation of portal veins to hepatic veins and it has been postulated that this is due to vascular obstruction. A study in non-alcoholic hepatitis has demonstrated that 20 % of hepatic veins were obstructed in fibrosis stage 0. This increased to 45 % in stage 3-4 fibrosis [10]. These figures suggest that vascular obstruction is an important pathological process even in the milder stages of disease. Furthermore, collapse of architecture and disruption of sinusoidal blood flow results in hypoxia of the hepatic parenchyma with resulting accentuation of inflammation and fibrosis (see section below on angiogenesis).

Inflammation

Inflammation involves the processes of cellular exudation and increased vascular permeability. The presence or absence of inflammation in the liver is usually determined by histology. Whilst the majority of chronic liver diseases are associated with inflammation, in others, e.g. haemachromatosis, histological evidence of inflammation may not be prominent. To try and subdivide fibrosis into "inflammatory" and "non-inflammatory" processes is over simplistic as the two processes have a great deal of overlap. Furthermore, histological examination offers a "snap shot" view of events and is also limited by biopsy size. Moreover, there may be evidence of release of cytokines associated with injury and inflammation even in the absence of a cellular infiltrate. Therefore, the absence of inflammatory cells on a biopsy does not always exclude an inflammatory aetiology. However by using this classification, one can highlight some of the proposed mechanisms for the development and progression of fibrosis.

The hepatic stellate cell (HSC) has a central role in the inflammatory process. Activation involves the transdifferentiation of the quiescent, retinoid storing HSC, into the activated, contractile 'myofibroblast'. In the activated state HSCs enter the cell cycle leading to an accumulation of HSCs in areas of injury. The activation and proliferation of stellate cells has a number of effects. Firstly, there is a direct increase in the amount of matrix produced, in particular collagen 1 but also collagen IV and collagen III. Secondly, the HSC is a source of both metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). These substances lead to the degradation of collagen (MMPs) or inhibit the degradation of collagen (TIMPs) and

therefore the relative production of these will influence fibrosis accumulation or degradation. Studies have demonstrated a positive correlation between the degree of fibrosis and the accumulation of activated HSCs in the damaged liver [11;12]. Conversely the resolution of acute liver injury following paracetamol has been shown to be associated with a reduction of activated HSCs [13]. The matrix components present in hepatic fibrosis include collagens (predominantly interstial but including other collagen components eg type IV collagen), proteoglycans and matrix glycoproteins. In turn this neo-matrix may regulate progression. The normal basement membrane type matrix (rich in collagen IV) and its replacement with a matrix rich in type 1 collagen, associated with inflammation and fibrosis, is likely to critically alter cell-matrix interactions resulting in activation and perpetuation of activation of HSCs.

Other cell types involved in the inflammatory process include Kupffer cells, and T lymphocyte cells, largely through cytokine mediators. T cell subtype has been demonstrated to be important; for example a Th2 response, in comparison to a Th1 response, promotes fibrogenesis [14]. This dichotomy of response may have evolved as a response to parasites, which classically elicit a Th2 driven response. There is also emerging interest in the relative contribution to fibrosis from other myofibroblasts i.e. portal myofibroblasts and myofibroblasts of bone marrow origin [15].

Contractility

The teleological reason for contractility is the necessity for the body to close wounds, an integral part of healing. In common with wound healing processes in other tissues, the myofibroblast population in hepatic areas determine contractile activity. The contraction of activated myofibroblasts may have a contributory role in the development of portal hypertension. Endothelin concentrations, a powerful stimulation for contraction of HSC, rise after fibrotic injury. To confound this, nitric oxide, which antagonises the effect of endothelin and is derived from endothelial cells, is reduced in injury. Receptors for endothelin-1 and other less potent factors including eicosanoids, prostaglandins, vasopressin, adenosine, thrombin, PAF and angiotensin 11 have been found on activated HSCs [16].

Angiogenesis

Sinusoids in the liver conduct blood form the portal tract to the central hepatic vein. Fenestration of the sinusoids allows hepatocytes to extract and secrete substances into the circulation. The endothelial lining of these sinusoids become non-fenestrated with the development of perisinusoidal fibrosis. This "capillarisation" of the sinusoids leads to functional shunting of the blood across the lobule with a resulting deficient supply of nutrients and oxygen. Relative hypoxia of the parenchyma can result in the release of angiogenic factors as evidenced by animal models [17;18]. The release of these factors may then contribute to the development of vascular structures within the fibrous tissue connecting portal to portal tract or more significantly, portal tract to central vein. Therefore both functional and anatomic shunting of blood occurs within the lobule, causing the development of a vicious circle encompassing hypoxia and fibrosis. Furthermore, vascular thrombosis also contributes to the vascular insufficiency. Imaging studies have demonstrated a significant proportion of portal vein thrombosis in cirrhotic patients [19-21] and when explanted livers of this group are examined at autopsy occlusion of the portal and hepatic vein are found in 36 and 70 % of patients respectively [22].

1.3 Clinical factors influencing progression of fibrosis

In general, the progression of fibrosis requires the presence of ongoing stimulus over months to years. There are exceptions to this including neonatal fibrosis and veno-occlusive disease which follow a more fulminant course, for reasons which are not entirely clear [23;24]. The rate of progression varies within and between diseases. In hepatitis C factors found to influence progression of fibrosis include male sex, duration of infection, acquisition of infection at an older age (>40), long term excessive alcohol intake, immunosuppression, Co-infection with HIV or HBV, and non-response to antiviral therapy. Additionally, longitudinal studies have suggested that the degree of necro-inflammation at the first biopsy may predict future fibrosis [25;26] and have added weight to the concept that fibrosis progresses in a non-linear fashion. In hepatitis B, ongoing inflammation, influenced by host and viral factors, correlates with fibrosis [27;28]. The risk factors for progression in NASH are still being defined but include obesity, insulin resistance and age[29].

Whilst some of the risk factors are intuitive, such as continuing inflammation and dual pathology, others are less so. In HCV and NASH, why does the age of acquisition of disease influence fibrosis progression? Is there a significant difference in the wound healing response with increasing age, perhaps determined by cellular senescence, or does this simply reflect a higher starting point due to sub-clinical fibrosis? Large studies to date have not shown that viral genotype has a role in fibrosis progression in HCV yet this clearly influences response to treatment [30]. There is however increasing evidence for the role of the host genotype in fibrosis e.g. polymorphisms including TGF- beta and angiotensinogen [31]. Recently, the genetic factor V leiden mutation has also been shown to be associated with rapid progression of fibrosis in hepatitis C [32].

The progression of fibrosis may therefore be through a series of common pathways but a multitude of factors determine to what extent and how quickly the final stage of cirrhosis is reached.

Reversibility of fibrosis

The reversibility of fibrosis has been demonstrated in a spectrum of diseases including autoimmune hepatitis treated with steroids, haemachromatosis responding to venesection, hepatitis C treated with antiviral therapy and biliary decompression for secondary biliary fibrosis [33-37]. The reversibility of cirrhosis is more contentious and it has been argued that for this to occur, there is a requirement to demonstrate complete regression of neo-vascularisation and architectural disturbance in addition to fibrosis [38].

1.4 Definition of cirrhosis

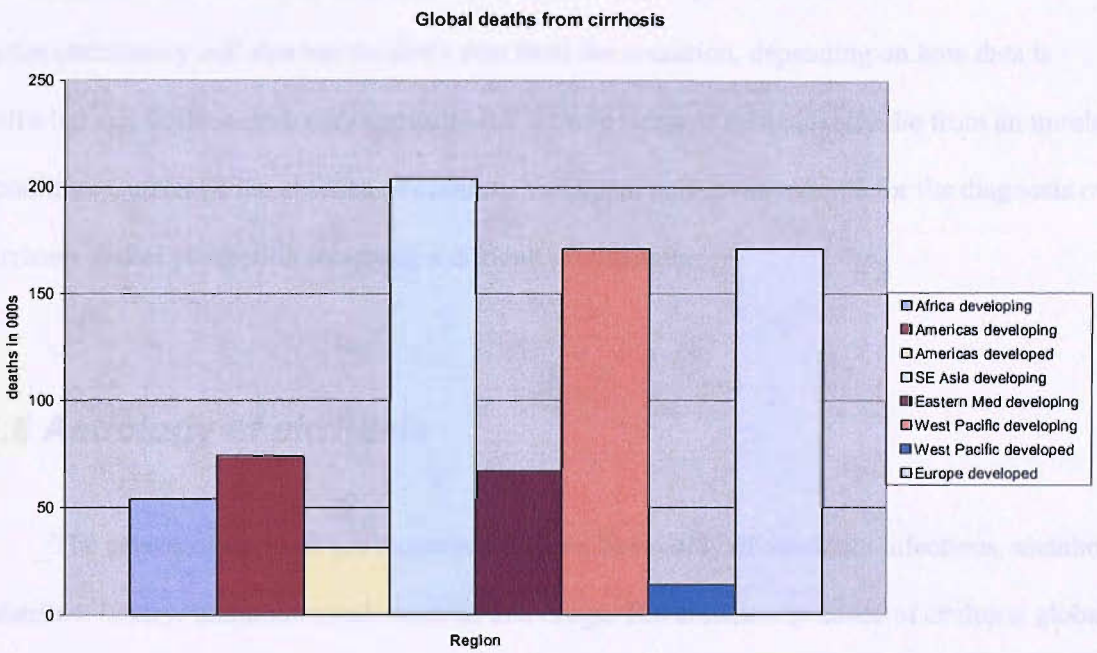
Cirrhosis is defined as the pathological findings of diffuse fibrosis and conversion of normal liver architecture into nodules. In the fifth century BC, Hippocrates recognised that hardening of the liver was a poor prognostic sign in the presence of icterus. The term cirrhosis is credited to Rene Laennec and derived from the Greek kirrhos, meaning orange-yellow. It described the autopsy appearance of the liver in a patient with cirrhosis which also demonstrated nodules and

an irregular edge. The definition has been refined over the years, notably by a consensus conference in La Habana 1956 and a working party sponsored by the World Health Organisation in 1978 [39], to provide us the basis for the present definition. Despite its morphological definition cirrhosis also implies a disturbance of hepatic and post hepatic haemodynamics which may play a significant role in the evolution of fibrosis and the clinical sequale.

1.5 Epidemiology

The number of deaths from cirrhosis in different regions is graphically illustrated below in figure 1.2. These figures are based on estimates made by the WHO report for 2002 [40].

Figure 1-2: Global deaths from cirrhosis



The temporal change of mortality from cirrhosis appears to vary world wide. A recent study examined cirrhosis mortality rates from 1980 to 2002 and found whist there was a decline in many regions including the USA, Japan and Australia there had been a rise in mortality in Eastern Europe and the UK [41]. Furthermore, a study in the Lancet demonstrated that mortality from cirrhosis almost doubled in Scotland and rose by 69 % in England and Wales, between 1997 to 2001, in contrast to reducing mortality in Southern Europe [42]. A major determinant of this is most likely related to a change in consumption and availability of alcohol. However, the contribution of obesity and viral

hepatitis as aetiological factors cannot be discounted until more accurate epidemiological data is made available.

The prevalence of cirrhosis is difficult to ascertain for a number of reasons. Firstly, cirrhosis is often clinically silent and a significant proportion of patients with undiagnosed cirrhosis have been found at autopsy studies [43]. Secondly, the reliance on death certification for the rates of cirrhosis is dependent on having the infrastructure to collect this data. Additionally, even if these resources are in place the stigmata of documenting cirrhosis and its aetiology will influence reporting.

Autopsy studies have an inherent bias because cirrhosis carries a risk of mortality. Furthermore, death rate may not always be a valid surrogate for prevalence and the relationship is not always linear. For example, if treatment significantly improves, the prevalence of the condition in the community will rise but the death rate from the condition, depending on how data is collected and documented, may appear to fall if these subjects subsequently die from an unrelated condition. Currently, the absence of accurate, validated, non-invasive tools for the diagnosis of cirrhosis makes population screening a difficult proposition.

1.6 Aetiology of cirrhosis

The causes of cirrhosis are numerous and can be broadly divided into infectious, metabolic, inherited, biliary, immunological, vascular and drugs. The commonest cause of cirrhosis globally is chronic hepatitis B. In the UK, the leading causes are alcohol, chronic hepatitis C (CHC) and Non-alcoholic fatty liver disease (NAFLD). As the latter two aetiologies form the basis of work in this thesis, they are described in greater detail below.

Chronic hepatitis C

Chronic Hepatitis C (CHC) is recognised as a major healthcare problem with a worldwide prevalence of over 200 million [44]. Prevalence is increasing, and estimates of the future

burden of CHC predict at least a three fold rise in cirrhosis by 2020 [45;46]. CHC can be acquired by contamination with biofluids and recognised routes of transmission include transfusion of blood products before 1991, intravenous drug abuse, tattooing and sexual intercourse. Approximately 70-85 % patients who acquire the virus develop chronic hepatitis C. The progression of chronic hepatitis C is variable and dependent on additional factors such as age of infection, gender and alcohol consumption. The majority of patients with CHC will develop hepatic fibrosis and between 25-30 % progress to cirrhosis with all the attendant long term complications of liver disease including death.

Hepatitis C is a RNA virus and diagnosis is suggested by the presence of antibodies to the virus and confirmed by measuring the virus in serum using the polymerase chain reaction method. Treatment for chronic infection involves pegylated interferon and ribavarin for either 24 or 48 weeks. The response rates depend on the family of virus, eg there is a sustained response in approximately 80-90 % of patients with genotype 2 or 3 but only 40-50 % in patients with genotype 1. Following evidence that the treatment of mild disease is cost effective [47] there has been a recent change in UK national guidelines to extend antiviral therapy to individuals with mild disease.

NAFLD

The link between liver disease and the metabolic syndrome, previously known as syndrome X, has become more clearly established over recent years. The metabolic syndrome is characterised by insulin resistance, obesity, hyperlipidemia and hypertension.

Non-alcoholic fatty liver disease (NAFLD) is emerging as one of the commonest causes of abnormal liver function tests and in the western world the estimated prevalence is reported to be as high as 30 % [3]. The prevalence of NAFLD is expected to rise in developed countries given the epidemic of its major underlying determinant obesity, in addition to the increasing ascertainment of this condition. Histologically and clinically NAFLD is a spectrum of disease from simple fatty deposition (steatosis), to necroinflammation in zone 3 in association with ballooning degeneration (non-alcoholic steatohepatitis (NASH)), to periportal and/or perisinusoidal fibrosis before eventually developing cirrhosis. The natural history is varied, some progress at varying rates to

cirrhosis, some remain stable at the same histological stage and grade and some have regression of disease or exist in variations of the above eg isolated portal fibrosis. The true incidence of cirrhosis related to NAFLD is difficult to know precisely because of the indolent and asymptomatic nature of the disease. Moreover, with advancing disease the steatosis is replaced by fibrotic tissue and therefore there may be few clues of NAFLD histologically. It is thought that a significant proportion of patients provisionally diagnosed with cryptogenic cirrhosis have underlying NAFLD. This is evidenced by studies showing a greater incidence of diabetes and obesity in cryptogenic cirrhosis compared to cirrhosis of other causes [48-50].

The exact pathophysiological mechanisms of NAFLD are incompletely understood. Insulin resistance and hepatic steatosis are thought to be significant independent factors; in addition they both have a deleterious effect on each other. Triglyceride accumulation is thought to result from excess free fatty acid influx into the liver. The "second hit" may come from a variety of sources, including oxidative stress, endotoxins and cytokines, and leads to necroinflammation.

The production of hormones and cytokines from fat depots, termed adipokines, is also thought to have a role in the evolution of NASH. Individuals with NASH have been shown to have low levels of adiponectin and high levels of TNF- alpha [51]. Whist TNF – alpha promotes insulin resistance and liver inflammation, adiponectin antagonises fatty acid oxidation and reduces the production and activity of TNF-alpha. In addition, adiponectin is antifibrotic. The hormone leptin has been shown to be important in the development of fibrogenesis in animal models. The fibrotic response, absent in leptin deficient mice, becomes apparent once leptin is restored by exogenous injection [52]. Higher serum leptin are also found in patients with NASH compared to controls [53] . Research continues into the pathogenic roles of the adipokines and how they interact in patients with NASH.

1.7 Clinical implications of cirrhosis

Cirrhosis may progress insidiously without clinical signs or symptoms, giving rise to the term compensated cirrhosis. Compensated cirrhosis can continue for a variable length of time before the development of complications, hepatocellular carcinoma or hepatic failure. The appearance of these states is referred to as decompensated cirrhosis. As stated earlier in this chapter, quantifying the numbers of asymptomatic cirrhotics is difficult because of the bias of autopsy studies and lack of non-invasive diagnostic tools. To illustrate the spectrum of clinical complications of cirrhosis some examples are discussed below.

Portal Hypertension

Portal hypertension is defined by an elevated portal pressure greater than the normal value of 1-5 mm Hg. In general, portal pressure becomes clinically significant above a level of 12 mm Hg. The major complications of portal hypertension include ascites, gastrointestinal haemorrhage and renal dysfunction. Of these, gastrointestinal haemorrhage is the most dramatic and occurs frequently. Prospective studies suggest that 90 % of patients with cirrhosis will develop oesophageal varices and a third of these will bleed [54]. Furthermore, gastrointestinal haemorrhage has been reported to carry a short term mortality rate as high as 50 %, in the group with most severe liver dysfunction [55].

Hypersplenism

This occurs in the context of portal hypertension and results in a variable pancytopenia with sequestration of red blood cells, white blood cells and platelets in the splenic tissues. This will affect oxygen delivery, coagulation and the immune response.

Renal Dysfunction

In cirrhosis there is abnormal renal handling of sodium and this in combination with abnormal haemodynamics results in a spectrum of disease culminating in hepatorenal syndrome

(HRS). The progression to hepatorenal syndrome is associated with splanchnic vasodilatation, increased cardiac output, decreased peripheral resistance and renal vasoconstriction. Prognosis is dependent on the subtype of HRS, as defined by the International Ascites Club[56], with Type 2 HRS developing more insidiously and associated with increased survival compared to Type 1. Treatment options include pharmacological intervention with albumin and vasopressors, TIPS, dialysis procedures and orthotopic liver transplantation (OLT).

Hepatopulmonary Syndrome

This is a disorder of pulmonary oxygenation occurring in the context of liver disease or portal hypertension. It is not exclusive to cirrhosis, but in this setting studies have shown · Hepatopulmonary syndrome (HPS) has a prevalence of 24 % and is an independent risk factor for mortality [57]. The pathophysiology is thought to relate to nitric oxide production as evidenced by increased levels of endothelial nitric oxide synthetase (enos) and inducible nitric oxide synthetase (inos) [58;59]. This may cause microvasular dilatation and contribute to intrapulmonary shunts with resultant hypoxaemia. Diagnosis involves the exclusion of other causes of cardiopulmonary disease and the demonstration of dependent hypoxaemia and pulmonary shunts using microbubble echocardiography. Hitherto, there have only been anecdotal, case reports or small uncontrolled trials of success with medical treatment. Orthotopic liver transplantation remains the only effective long term treatment [60;61], although HPS will also increase the anaesthetic risks associated with transplantation

Cirrhotic cardiomyopathy

The affect of alcohol and iron on cardiac function have long been recognised. More recently, there has been increasing evidence of the effect of cirrhosis per se on the myocardium and the term "cirrhotic cardiomyopathy" was described in 1989 by Lee [62]. The features include abnormal baseline cardiac output, attenuated systolic contractility and diastolic relaxation in response to inotropic and chronotropic stimuli and the absence of florid left ventricular failure. There can be associated electrophysiological abnormalities such as repolarisation anomalies but morphological changes are often not pronounced. The exact pathophysiology is not completely understood but

possibilities include alterations in beta-adrenergic receptors, membrane function, calcium channels, nitric oxide and carbon monoxide [63]. Orthotopic liver transplantation has been shown to improve this condition [64].

Hepatocelluar carcinoma

Cirrhosis remains the commonest cause for the development of hepatocellular carcinoma (HCC); it has been estimated that 80% of tumours occur in this setting [65]. The aetiology and the global location of disease are important determinants of the risk of cirrhosis progressing to HCC. For example, the annual risk of developing HCC in patients with HCV is estimated to be between 3-8 % [66]. The 5 year cumulative incidence of HCC on the background of HCV is reported to be as high as 30 % in Japan compared to 17 % in the west. Comparing aetiologies, the rates decrease in the following order: Haemachromatosis, hepatitis B infection, alcohol and biliary disease [67]. Interestingly, HCC is an uncommon complication of autoimmune hepatitis [68]. Regardless of aetiology independent risk factors for HCC include gender, age, severity of compensated cirrhosis at presentation and sustained activity of liver disease [69].

Encephalopathy

Hepatic encephalopathy on the background of cirrhosis can occur in a sub-clinical, acute or chronic setting. The prevalence sub-clinical encephalopathy (SCE) in patients with cirrhosis has reached 84 % in some studies [70]. As there may be no compromise in routine daily life, SCE may only come to light upon psychometric testing using number connection tests, block reception tests and reaction times. Acute hepatic encephalopathy can be precipitated by sepsis, sedatives, dehydration, haemorrhage, etc. but it may also occur with no obvious precipitants in the presence of terminal liver failure. Chronic encephalopathy is usually due to the formation of porto-systemic shunts, as part of the disease or iatrogenically as a consequence of transjugular intrahepatic porto-systemic shunt (TIPS) insertion.

Nutrition

Cirrhosis is a catabolic state and in combination with dietary insufficiency leads to significant malnourishment. Additionally, malabsorption, hypermetabolism and resistance to growth factors such as insulin and growth factor contributes to the pathophysiology In hospitalised patients with liver disease the prevalence of malnourishment is estimated to be as high as 20 % in compensated disease rising to 60 % with severe liver insufficiency [71]. Studies have shown that nourishment, measured by parameters such as skin fold thickness and midarm muscle circumference is an independent predictor of survival at two years [72].

Measures of anthropometry, such as skin-fold thickness, may be distorted by fluid retention and occur late in malnutrition. Thus, there has been interest in finding accurate tools for assessing nutritional status. This has led to the creation of measures such as subjective global assessment tool (SGA), prognostic nutritional index (PNI) and hand grip strength (HGS). A recent study found that the prevalence of malnutrition was 63 % in patients with Childs A cirrhosis using HGS, compared to a prevalence of 28 % and 19 % detected by SGA and PNI respectively [73].

Sepsis

It has been estimated that the incidence of sepsis in cirrhosis is 30 to 50 % of hospital admissions [74]. Once sepsis has developed there is a higher rate of mortality, up to 30 %, and this is independent from the severity of liver disease [75;76].

There are a number of reasons for increased susceptibility to sepsis in this group. Bacterial translocation (BT) represents the migration of bacteria or their products from the intestinal lumen to mesenteric lymph nodes. BT has been shown to be significantly increased in patients with Childs C cirrhosis compared to the less severe Childs' stages [77]. Moreover, the amount of antigen load may not only increase but changes of immunity, within the local lymph nodes and systemically, will also determine if there is progression to sepsis. Studies have shown a reduced phagocytic and killing capacity of the immune system [78;79] and reduced opsonisation relating to low complement levels in ascites [80].

1.8 Diagnosis of liver fibrosis and cirrhosis

Simple clinical, haematological and biochemical parameters may only alter once severe disease has been established. Even in the presence of established cirrhosis, these parameters can remain unchanged. For example, although usually elevated in cirrhosis, both AST and ALT can be normal in up to 10 and 35 % cases respectively [81]. The addition of radiology can improve the detection of cirrhosis, diagnostic accuracy rates of 80-90 % have been published using ultrasound [82], but presently traditional imaging modalities are unable to detect fibrosis with acceptable diagnostic accuracy.

Histology

Histological analysis remains the "gold standard", and arguably from the purist definition, the only method of diagnosing cirrhosis. Various histological classifications exist for the grading of fibrosis and cirrhosis including Sheuer, Ishak and Metavir (see appendix 1); the majority of scores were originally validated for hepatitis C.

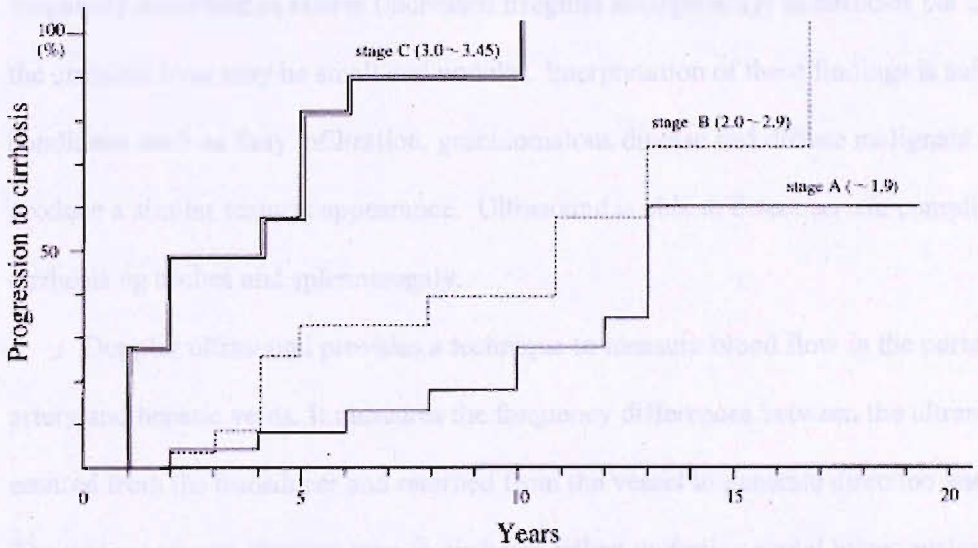
The method for obtaining a liver biopsy can vary from the blind versus image guided transabdominal approach, transjugular approach, laparoscopic approach and open approach at operation or post-mortem. The choice of route will depend on factors such as body habitus, clotting abnormalities, the presence of ascites, available expertise and the quantity of specimen required.

The biopsy provides information in three major areas. Firstly, it can elucidate aetiology eg marked accumulation of iron in the hepatic parenchyma suggests haemachromatosis whereas biliary duct damage associated with granulomas raises the suspicion of primary biliary cirrhosis. Secondly, it provides information on underlying pathological processes eg steatosis, necroinflammation and fibrosis and stages the severity of disease eg the presence of cirrhosis. Thirdly, it can also act a prognostic indicator. For example, in the context of hepatitis C, inflammation in the biopsy is suggestive of future fibrosis [83].

The liver biopsy has been useful in providing information on the natural history of the progression of fibrosis. Yano et al performed a longitudinal study on 70 patients with chronic

hepatitis C [84]. The patients had between two to ten liver biopsies between 1 to 26 years (mean 9 years). Fibrosis at the initial biopsy (index biopsy) was divided into three stages, A (none/mild), B (moderate) and C (severe). The progression to cirrhosis is depicted in figure 1.3 below and demonstrates that increased fibrosis at the index biopsy increases the rate of progression and provides evidence that the evolution of fibrosis to cirrhosis may not occur in a linear fashion.

Figure 1-3: Progression of fibrosis to cirrhosis (Yano et al)



Liver biopsy does have its limitations and some have questioned whether it truly represents a "gold standard" reference test. In large studies of patients undergoing biopsy pain has been reported in 20 % and severe complications reported in 0.57 % [85] . Sampling error exists, which is unsurprising considering the average biopsy specimen represents1/50 000th of the organ. Studies have shown discordance rates of up to 30 % when right and left lobes are sampled at laparascopically, even in homogenously distributed disease [86]. Interpretation of the biopsy is open both to intra and inter observer error [87;88] . Finally, classification of histological scores into categorical variables may not truly reflect the dynamic progression and regression of fibrosis and the use of numerical scores can give a misleading picture of fibrosis progression.

The biopsy offers a wealth of information but for the measure of fibrosis, in the individual and population, better tools may be required.

Imaging techniques

Ultrasound

Ultrasound is a relatively non-invasive method of assessing the size, texture, vascular patency and for detecting space occupying lesions in the liver. The former parameters may change in cirrhosis, but unfortunately are not specific to the condition. The cirrhotic liver may be enlarged (eg non-alcoholic steatohepatitis), normal or reduced in size. The echo pattern of the liver is frequently described as coarse (increased irregular echogenicity) in cirrhosis but can alternatively the cirrhotic liver may be small and nodular. Interpretation of these findings is subjective and conditions such as fatty infiltration, granulomatous disease and diffuse malignant infiltration can produce a similar textural appearance. Ultrasound is able to detect certain complications of cirrhosis eg ascites and splenomegaly.

Doppler ultrasound provides a technique to measure blood flow in the portal vein, hepatic artery and hepatic veins. It measures the frequency differences between the ultrasound signal emitted from the transducer and returned from the vessel to generate direction and velocity of flow. The major vascular changes seen in cirrhosis reflect underling portal hypertension. They include reversal of portal vein blood from hepatopetal to hepatofugal, flattening of the doppler waveform in hepatic veins and enlargement of the portal vein to greater than 15 mm.

Ultrasound has a further role in detecting the development of hepatocellular carcinoma on the background of cirrhosis. Isolated nodules, larger than 2 cm are detected with a good sensitivity but a lower specificity. In combination with an elevated alpha-fetoprotein the presence of HCC can be suspected. The performance of ultrasound in the presence of diffuse malignancy is poor.

Computed Tomography (CT)

CT also has a role in assessing parenchymal disease in cirrhosis. The density of liver parenchyma is usually within the normal range but can vary depending on aetiology; eg increases in haemachromatosis and decrease in fatty infiltration. The advantage of CT is that is provides

cross sectional anatomy. With the advent of multi slice CT scanning detailed information about space occupying lesions can be gleaned. Furthermore, the addition of contrast agents such as lipiodal may improve detection of HCC. Helical CT can also utilise vascular anomalies of lesions by obtaining images in the arterial or portal phase. For example HCC typically will have a brisk enhancement during the arterial phase but appears hypodense during the portal phase. Direct catheterisation of the superior mesenteric artery and hepatic artery can also be used in combination with CT to improve diagnostic accuracy. Therefore, CT in isolation or combination with arteriography is able to provide a "road map", highlighting the blood supply of lesions or vascular anomalies that occur in the context of cirrhosis. The role of CT in diagnosing fibrosis is yet to be established and one obstacle for its widespread use may be the associated radiation exposure. The rapidly evolving technology and reduced acquisition times associated with multi slice CT may address some of these issues.

MRI

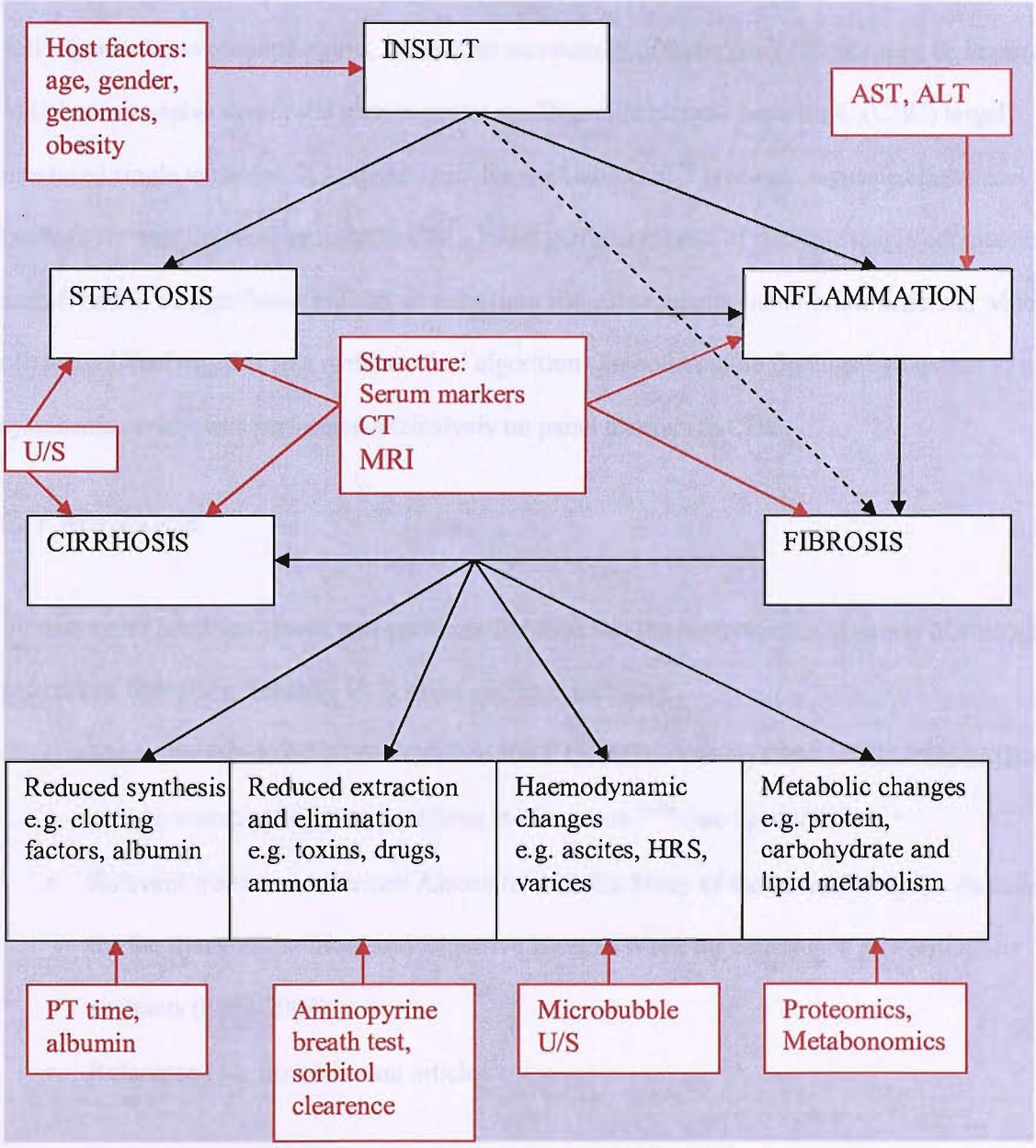
This modality represents an emerging tool in liver disease. Dramatic and remarkably clear images of cirrhosis can be obtained in advanced disease. There is evidence for the use of MRI in assessing hepatic iron concentration [89] and differentiating small benign nodules and HCC [90]. Injection of resovist may help distinguish lesions of focal nodular hyperplasia and HCC. MRI spectroscopy is currently an experimental tool but may yet emerge to provide a measure of metabolic changes within the liver.

1.9 The role of biomarkers in the assessment of liver fibrosis

As discussed above liver fibrosis is a result of chronic injury to the liver and the progression to cirrhosis is associated with changes in a number of key functions of the liver; Figure 1.4 represents a simplified summary. Finding biomarkers that assess liver fibrosis could be derived from a number of broad areas and the boxes in red represent examples of this. Host factors which interact with the insult will determine if and how quickly fibrosis will progress (eg age, sex and genetic polymorphisms). Other potential sources of biomarkers are measures of pathological

processes preceding fibrosis (eg ALT as a measure of necroinflammation). Directly measuring structural alterations associated with fibrosis (eg collagen turnover products measured by serum markers or imaging techniques such e.g. ultrasound, computerised tomography, etc) is analogous to histological assessment. Finally, biomarkers measuring the functional consequence of fibrosis (eg reduced clotting and albumin) will be of clinical relevance for the planning of intervention such as liver transplantation.

Figure 1-4: Simplified summary of the causes and consequences of liver fibrosis and potential areas of biomarker development.



Chapter 2 : Systematic review of panel markers to assess fibrosis in chronic hepatitis C

Background

The diagnosis of liver fibrosis in chronic hepatitis C is of importance for a number of reasons. It will identify patients who are in greatest need of treatment and influence the timing of treatment. Furthermore, the diagnosis of severe fibrosis/cirrhosis will have implications on duration of therapy, future therapeutic strategies and the surveillance of the complications of cirrhosis. Historically, the liver biopsy has been prerequisite for the initiation of antiviral therapy in the UK. Recent NICE guidelines [91] do allow the initiation of therapy without a liver biopsy but there are still arguments, as outlined above, for why an assessment of underlying fibrosis may be important. Initial non-invasive diagnostic tests in assessing fibrosis in chronic hepatitis C (CHC) largely measured single variables. A systematic review by Gebo et al [92] however suggested that panel markers showed the most promise in CHC. Panel markers consist of multiple single components, each found to be significant initially at univariate and subsequently multivariate analysis, which are often combined together in a mathematical algorithm. To build on the findings by Gebo et al a systematic review was performed exclusively on panel markers in CHC.

2.1 Methods

A systematic literature search was performed to ascertain the performance of panels of surrogate markers of fibrosis in Hepatitis C. Sources searched included:

- Electronic databases 1985 – October 2004: Cochrane Library 2004 MEDLINE, EMBASE using a search strategy derived from the literature [93;94] (see appendix 2).
- Relevant websites: American Association for the Study of the Liver, European Association for the Study of the Liver and Digestive Disease Week for conference proceedings or abstracts (2002-2004).
- Reference lists from relevant articles.

Inclusion criteria

Studies were included if;

- they evaluated panels of ≥2 serum markers
- allowed extraction of data for interferon naïve patients with CHC
- were written in English
- were systematic reviews, meta-analyses or studies of diagnostic tests
- they used liver biopsy as a reference standard.
- they included >30 participants (as smaller studies will be underpowered to produce precise estimates of test performance and would be more likely to produce zero denominator effects in 2x2 table).

A serum marker was defined as any measure that could be derived from a blood sample. Studies identified by the search strategy were assessed for inclusion by two reviewers (NG, JP).

Exclusion criteria

Studies were excluded if;

- data on hepatitis C were not separately extractable
- study did not produce a composite score

Data extraction strategy

Data extraction was undertaken by one reviewer (NG) and checked by a second reviewer (JP) with any disagreements being resolved through discussion. A third reviewer was consulted (PR) to resolve persisting issues. Information collected included patient demographics, test assay details, background prevalence of fibrosis severity, risk factors, histological parameters, statistical methods used, and test performance characteristics.

Quality assessment strategy

The quality of included studies was assessed using the quality assessment of diagnostic accuracy studies (QADAS) tool [95]. (Appendix 3)

Data analysis/synthesis

Data are presented with full tabulation of results of included studies. Where data were available, 2x2 tables were constructed to derive sensitivity, specificity, predictive values, likelihood ratios (LR) and diagnostic odds ratios (DOR) at each threshold value. Accepted levels for robust tests are - LR = <0.1, and +LR = >10, >5 and <0.2 give strong diagnostic evidence. For DOR reasonable test performances would be >30 [96]. The percentage of patients in each study to which the different thresholds could be applied were derived where possible. The methodology used by investigators to create the various diagnostic algorithms is shown in table 2.4. We evaluated the performance of tests at thresholds which produced clinically useful predictive values with acceptable false negative and positive rates, based on local clinical opinion (NPV ≥95% PPV ≥90%) as there are is no consensus on robust predictive values for the diagnosis of liver fibrosis. Using this method we calculated how many liver biopsies could be avoided appropriately, with patients below NPV of <95% assumed to have no significant fibrosis and patients with PPV >90% assumed to have significant fibrosis. The performance of tests was calculated for different fibrosis stages- early versus moderate/severe fibrosis (F0/F1 vs F2/F3/F4) and also for cirrhosis or no cirrhosis (F0/F1/F2/F3/vs F4).

2.2 Results

The electronic search yielded 2,766 abstracts which were read in full. 25 full papers were retrieved of which 9 were excluded leaving 14 studies in separate populations to be included in the review (see Table 2.1). Reasons for exclusion were;

- single markers 4 papers
- less than 30 participants 4 papers
- no reference test 1 paper

In addition two reviews were identified; a systematic review of all serum markers and an overview of two markers (Fibrotest and Actitest) [97;98]. All relevant studies reported in these reviews have been included in this review. Primary data from several studies presented in the Fibrotest/Actitest review but not reported elsewhere were utilised in the review.

Many of the studies reported >10 /14 of the QUADAS criteria, with homogeneity of criteria met or unmet (see appendix 4). Ten different panels of serum markers were reported. Ten studies reported sufficient information to derive sensitivity, specificity, predictive values, DOR and LR at specific cut-offs. Patient characteristics varied between studies, median age was 44.5 years (range 39-47 yrs), and the median proportion of male subjects was 64% (range 45-71%). Four studies presented CHC risk factors. The proportion with moderate/severe fibrosis (F2 F3 F4) was 43% (median) with a range of 17-80%. The fibrosis staging systems used to classify the histology varied, Metavir (7), Scheuer (4), Ishak (1), Knodell (1), Desmet,(1) and local scoring system (1). Quality of liver biopsy as assessed by number of portal tracts, and length of biopsy was reported in 7 studies, with 3 having both these criteria. Study design was similar in most studies, with paired histology and serum samples on individual patients with untreated CHC being analysed retrospectively from an existing cohort (n=7), or prospectively recruited (n=8) and then analysed at a single point in an individual's illness. In 6 studies recruitment was consecutive. 11 studies presented data validated in a different group of patients than the training set including five studies of patients recruited at the same centre as the training cohort (internal validation) and six studies that recruited subjects at a different centre (external validation)

Table 2-1: Characteristics of studies evaluating the performance of panels of serum markers of liver fibrosis: F0/1 vs F2/3/4

Author Year Country (n centres)	Total Patients in study	Serum marker tests in panel	Test used to compare	Validity sample (n)	Patient selection	Age mean (yrs)	% male	% severe Fibro-sis	% IDU	Liver biopsy scoring system	Biopsy Mean Length (L) Portal Tracts (PT) Observers (O)
Imbert-Bismut (2001)[99] France (1)	339	AST, ALT, albumin, α_1 globulin, β globulin, γ globulin, bilirubin, GGT, α_2 macroglobulin, haptoglobulin, apolipoprotein A1. Combinations 10, 6, &5 markers	n/r	Training set (n=205) Internal validation set (n=134)	*DOSVIRC cohort Prospective recruitment	47	58	40	n/r	Metavir	>10 mm (L) n/s (PT) 1 (O)
Rossi (2003)[100] Australia (1)	125	bilirubin, GGT, α_2 macroglobulin, haptoglobulin, apolipoprotein A1 corrected for age and sex.(Fibrotest)	n/r	Whole study = external validation of test	Consecutive prospective recruitment	40	66	38	n/r	Metavir	n/s (L) n/s (PT) 1 (O)

Poynard (2003)[101] Europe Argentina Canada USA (62)	352	FT-AT (Fibrotest + ALT)	Fibrotest	Whole study = external validation of test	From RCT treatment (n=1530) Retrospective recruitment	45	64	17	n/r	Metavir & Knodell	17 mm (L) >6 mm in 89 % (PT) 1 (O)
Wai (2003)[102] USA (1)	270	AST: platelet ratio (APRI)	n/r	Training set (n=192) Internal validation set (n=78)	Treatment naive Retrospective recruitment	46	64	64	41	Ishak	n/s (L) n/s (PT) 1 (O)
Le Calvez (2004)[103] France (1)	323	AST: platelet ratio (APRI)	Fibrotest	Whole study = external validation of test	*DOSVIRC cohort Retrospective recruitment	n/r	n/r	41	n/r	Metavir	> 10 mm (L) n/s (PT) n/s (O)
Forns (2002)[104] Spain (1)	476	Age, GGT, cholesterol, platelets,	n/r	Training set (n=351) Internal validation set (n=125)	Consecutive prospective recruitment	39	64	25	n/r	Sheuer	n/s (L) >6 (PT) 1 (O)
Thabut (2004)[105] France (1)	249	Forns index score	Fibrotest	Whole study = external validation of test	*DOSVIRC cohort Retrospectivre cruitment	n/r	n/r	38	n/r	Metavir	> 10 mm (L) n/s (PT) n/s (O)

Sud (2004)[106] Australia (2)	302	Fibrosis probability index =age, AST, HOMA-IR (fast glucose x plasma gluc/22.5), total cholesterol, past etoh consumption.	APRI Wai	Training set (n=176) Internal validation set (n=126)	Consecutive Prospective recruitment patients having biopsy without cirrhosis	41	56	54	61	Sheuer	n/s (L) n/s (PT) n/s (O)
Leroy (2004)[107] France (1)	388	PIIINP/MMP-1/HA/MMP-2/MMP-9/TIMP-1/TIMP-2	PIIINP HA	Training set (n=194) controls(n=194)	Consecutive prospective recruitment	43	64	45	40	Metavir	19 mm (L) 14 (PT) 1 (O)
El Shorbagy (2004)[108] Egypt (1)	109	Platelets, MMP-9, portal vein diameter, splenic longitudinal axis, ALT, AST, viral load.	n/r	Training set (n=109)	CHC patients from general population screening	47	71	80	n/r	Local scoring system	n/s (L) n/s (PT) n/s (O)
Patel (2004)[109] USA France (4)	696	HA, TIMP-1, α2 macroglobulin	HA TIMP1, α2 macroglobulin	Training set (n=294) External validation set (n=492)	Retrospective selection made on equal numbers F0-F4	45	69	51	n/r	Metavir	13 mm (L) >5 (PT) 1 (O)
Rosenberg (2004)[110] Europe (13)	325	3 marker panel – age, HA, PIIINP, TIMP-1.	n/r	Training set (n=164) External validation set (n=261)	Prospective recruitment	44	63	27	n/r	Sheuer	>12 mm (L) >5 (PT) 1 (O)

Table 2-2: Characteristics of studies evaluating the performance of panels of serum markers of liver fibrosis: F0/1/2/3 vs F4

Author Year Country (n centres)	Total Patients in study	Serum marker tests in panel	Test used to compare	Validity sample (n)	Patient selection	Age mean (yrs)	% male	% severe Fibro-sis	% IDU	Liver biopsy scoring system	Biopsy Mean Length (L) Portal Tracts (PT) Observers (O)
Kaul[111] 2002 USA (2)	264	Probability model – Platelets, AST, sex, spider naevi.	n/r	Training set (264) External validation set (102)	Retrospective recruitment	n/r	45	61	33	Sheuer	n/s (L) n/s (PT) 1 (O)
Fortunato (2001)[112] Italy (1)	103	Fibronectin, prothrombin, pseudocholinestera se, ALT, manganese superoxide dismutase N-acetyl β – glucosaminidase.	n/r	Training set (63) Internal validation set (40)	Prospective recruitment	n/r	n/r	n/r	n/r	Desmet	n/s (L) n/s (PT) >2 (O)

Table 2-3: Results of studies evaluating the performance of panels of serum markers of liver fibrosis F0/1 vs F2/3/4

Study and year of publication	Cut off levels reported	Cumulative %		Sensitivity		Specificity		PPV		NPV		+ VE LR		-VE LR		AUC	
FIBROTEST		T	V	T	V	T	V	T	V	T	V	T	V	T	V	T	V
Imbert-Bismut(2001) ***	0.1	16	12	97	100	24	22	44	51	93	100	1.3	1.3	0.1	0		
	0.3	48	39	79	87	65	59	58	63	84	85	2.3	2.1	0.3	0.2	0.84	0.87
	0.6	77	66	51	70	94	95	84	91	76	80	8.6	12.9	0.5	0.3		
	0.8	86	81	29	38	95	97	78	92	69	66	5.7	14.2	0.7	0.6		
Rossi (2003)	0.1	n/a	21	n/a	92	n/a	29	n/a	45	n/a	85	n/a	1.3	n/a	0.3		
	0.2	n/a	38	n/a	83	n/a	52	n/a	52	n/a	83	n/a	1.7	n/a	0.3		
	0.3	n/a	47	n/a	75	n/a	61	n/a	54	n/a	80	n/a	1.9	n/a	0.4		
	0.4	n/a	61	n/a	67	n/a	78	n/a	65	n/a	79	n/a	3	n/a	0.4		
	0.5	n/a	69	n/a	56	n/a	85	n/a	70	n/a	76	n/a	3.6	n/a	0.5	n/a	0.74
	0.6	n/a	80	n/a	42	n/a	94	n/a	78	n/a	72	n/a	6.4	n/a	0.6		
	0.7	n/a	82	n/a	35	n/a	94	n/a	77	n/a	70	n/a	5.4	n/a	0.7		
	0.8	n/a	89	n/a	22	n/a	96	n/a	79	n/a	66	n/a	5.8	n/a	0.8		
	0.9	n/a	95	n/a	8	n/a	97	n/a	57	n/a	63	n/a	3.2	n/a	0.9		
Poynard (2003) (Before Treatment)	0.1	n/a	6	n/a	97	n/a	80	n/a	41	n/a	81	n/a	1.1	n/a	0.4		
	0.3	n/a	33	n/a	86	n/a	45	n/a	50	n/a	83	n/a	1.6	n/a	0.3	n/a	0.73
	0.6	n/a	67	n/a	50	n/a	79	n/a	61	n/a	71	n/a	2.4	n/a	0.6		
	0.8	n/a	89	n/a	20	n/a	95	n/a	72	n/a	65	n/a	3.9	n/a	0.8		

APRI																	
Wai (2003)	0.5 1.5	29 78	n/a n/a	91 41	n/a n/a	47 95	n/a n/a	61 88	n/a n/a	86 64	n/a n/a	1.7 8.2	n/a n/a	0.2 0.6	n/a n/a	0.8	0.89
Le Calvez (2004) (APRI VAL) ***	0.5 1.0 1.5 2	n/a n/a n/a n/a	41 68 80 87	n/a n/a n/a n/a	81 54 36 24	n/a n/a n/a n/a	56 84 91 95	n/a n/a n/a n/a	56 70 73 76	n/a n/a n/a n/a	81 73 68 65	n/a n/a n/a n/a	1.8 3.3 4.1 4.5	n/a n/a n/a n/a	0.3 0.5 0.7 0.8	n/a	0.74
Sud (2004)	n/r	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.76
FORNS																	
Forns (2002)	4.2 6.9	36 87	39 88	94 44	94 30	45 96	51 95	35 79	40 66	96 84	96 80	1.7 11.6	1.9 6.0	0.1 0.6	0.1 0.7	0.86	0.81
Thabut (2004) (FORNS VAL) ***	1 3 6 8	n/a n/a n/a n/a	2 16 70 91	n/a n/a n/a n/a	1 1 55 19	n/a n/a n/a n/a	4 26 86 97	n/a n/a n/a n/a	39 45 70 78	n/a n/a n/a n/a	1 1 75 66	n/a n/a n/a n/a	1 1.4 3.8 5.8	n/a n/a n/a n/a	0 0 0.5 0.8	n/a	0.78

*** from comparative hepatology Poynard T. et al 2004[18]

Sud (2004) (FORNS VAL)	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	0.76
Sud (2004)	0.1	14	16	100	91	26	25	56	63	100	65	1.4	1.2	0	0.4		
	0.2	24	29	96	85	43	48	61	70	93	69	1.7	1.6	0.1	0.3		
	0.3	32	40	93	74	54	60	65	73	89	62	2	1.8	0.1	0.4		
	0.4	42	48	87	68	69	69	72	76	85	60	2.8	2.2	0.2	0.5		
	0.5	52	55	73	64	74	81	72	83	75	61	2.8	3.3	0.4	0.5	0.84	0.77
	0.6	57	60	58	58	83	85	76	84	68	58	3.4	3.8	0.5	0.5		
	0.7	72	70	49	49	91	96	83	95	66	57	5.6	12.6	0.6	0.5		
	0.8	76	75	43	42	94	98	87	97	64	54	6.6	21.8	0.6	0.6		
	0.9	87	88	27	19	100	98	100	93	60	46	E	9.8	0.7	0.8		
Leroy (2004)	0.2	24	n/a	91	n/a	35	n/a	55	n/a	88	n/a	1.5	n/a	0.2	n/a		
	0.3	61	n/a	65	n/a	85	n/a	76	n/a	75	n/a	4.3	n/a	0.4	n/a	0.82	n/a
	0.4	82	n/a	35	n/a	96	n/a	91	n/a	65	n/a	8.6	n/a	0.7	n/a		
	0.5	93	n/a	17	n/a	99	n/a	100	n/a	60	n/a	33.1	n/a	0.8	n/a		
El Shorbagy 2004	0-3	32 ^	n/a	82	n/a	80	n/a	51	n/a	95	n/a	4.2	n/a	0.2	n/a		
	4-6	55 ^	n/a	69	n/a	67	n/a	77	n/a	57	n/a	2.1	n/a	0.5	n/a	0.8	n/a
	6-9	17 ^	n/a	80	n/a	97	n/a	84	n/a	96	n/a	24	n/a	0.2	n/a		
Patel (2004)	0.36	41	47	83	77	66	73	72	76	79	75	2.4	2.9	0.3	0.3		
ELF Rosenberg (2004)	0.063			n/a	95	n/a	29	n/a	28	n/a	95	n/a		n/a			
	0.067			n/a	90	n/a	31	n/a	28	n/a	92	n/a		n/a			
	0.09			n/a	85	n/a	43	n/a	30	n/a	91	n/a		n/a			
	0.126			n/a	80	n/a	58	n/a	35	n/a	91	n/a		n/a			0.77
	0.190			n/a	63	n/a	80	n/a	48	n/a	89	n/a		n/a			
	0.219			n/a	52	n/a	85	n/a	50	n/a	86	n/a		n/a			
	0.268			n/a	47	n/a	90	n/a	58	n/a	86	n/a		n/a			
	0.426			n/a	38	n/a	95	n/a	70	n/a	84	n/a		n/a			
	0.564			n/a	30	n/a	99	n/a	90	n/a	83	n/a		n/a			

Table 2-4: Results of studies evaluating the performance of panels of surrogate markers of liver fibrosis F0/F1/F2/F3 vs F4

Study and year of publication (date of study) country (no centres)	Cut off levels reported	Sensitivity		Specificity		PPV		NPV		+ VE LR		-VE LR		AUC	
FIBROTEST		T	V	T	V	T	V	T	V	T	V	T	V	T	V
Imbert-Bismut(2001) ***	<0.8 >0.8	n/r	n/r	n/r	n/r	85	n/r	90	n/r	n/r	n/r	n/r	n/r	0.92	n/r
Poynard (2003)		n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	0.73
APRI															
Wai	<0.1 <0.2	89 57	n/r n/r	75 93	n/r n/r	38 57	35 65	98 93	100 95	n/r n/r	n/r n/r	n/r n/r	n/r n/r	0.89	0.94
Le Calvez		n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	0.80
PIIINP MMP1															
Leroy	0.2 0.3 0.4 0.5	94 85 58 26	n/r n/r n/r n/r	28 74 98 97	n/r n/r n/r n/r	n/r 43 66 77	n/r n/r n/r n/r	95 95 91	n/r n/r n/r n/r	n/r n/r n/r n/r	n/r n/r n/r n/r	n/r n/r n/r n/r	n/r n/r n/r n/r	0.88	
El Shorbagy		n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	0.80	
Platlets/spiders/sex/ AST															
Kaul		n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	n/r	0.93	0.93

Table 2-5: Examples of methodology used to create the different panel markers.

Panel Marker Test	Statistical analysis used to create final algorithm	Final algorithm produced
Fibrotest	Logistic regression and artificial neural network	Score: -5.540 + 4.467* log (alpha-2 macroglobulin) – 1.357* log(haptoglobulin) + 1.017*log (GGT) +0.0281* (age) + 1.1737*log (bilirubin)-1.184*(apo A1) + 0.301*sex(female =0, male=1)
APRI	Multivariate forward logistic regression	(AST/ULN)/ Platelets * 100
Forns	Multivariate forward stepwise logistic regression	Score: 7.811-3.131.ln(platelet count) + 0.78.ln(GGT) + 3.467.ln(age)-0.014.(cholesterol)
HOMA-IR	Multivariate forward logistic regression	Score: fasting glucose * (plasma glucose/22.5)
Fibrospect	Logistic regression and discriminant function analysis.	HA, TIMP-1 and alpha-2 macroglobulin- algorithm not published
ELF	Discriminant analysis	Score: -6.38 - (ln(age)*0.14) + (ln(HA)*0.616) + (ln(P3NP)*0.586) + (ln(TIMP1)*0.472).

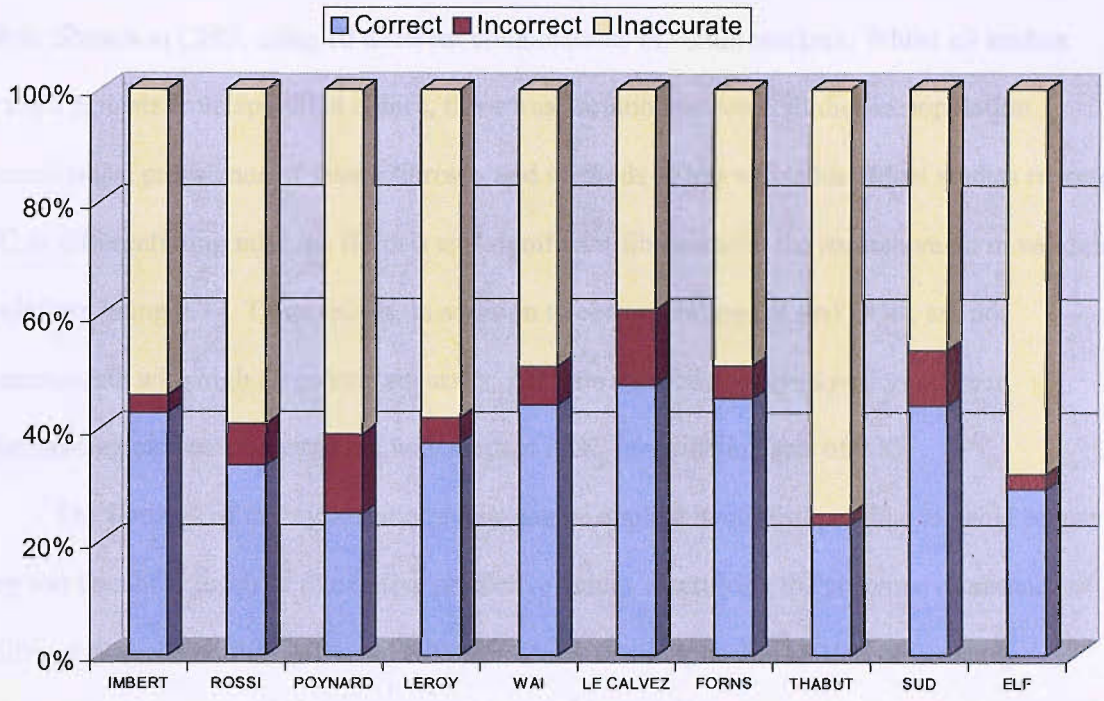
Results from studies differentiating (F0/F1 vs F2/F3/F4)

For those studies presenting data on no/mild versus moderate/severe fibrosis, 10 studies presented data at several thresholds and had sufficient information to permit the derivation of true positive, true negative, false positive, and false negative (Table 2.3). The number of thresholds presented for each test varied. AUC for Receiver Operating Characteristic (ROC) curves were presented in 13/14 studies (Table 2.3). Median AUC for training sets was 0.81 (range 0.80 to 0.84), and for the validation sets 0.77 (0.73 to 0.90). Likelihood Ratios (LR) and diagnostic odds ratios (DOR) were derived for 10 studies. Negative LR ranged from 0.1 to 0.9 (– LR), and positive LR 1.2 to 33.1 (+LR). DOR was 9.0 (median) with a range of 5 to 27. The cumulative percentage of patients to whom the panel score was applicable at each cut-off is presented (Table 2.3). The proportion of people at thresholds where the PPV ≥90% and NPV ≥95% was 40% (training set), 29% (validation set), overall 35% (median values).Clearly this value will rise if one lowers the predictive values used.

At each threshold tests perform with either high sensitivity with low specificity or vice-versa. The summative DORs are all <10. Figure 2.1 shows the results of modelling where

thresholds giving PPV of approximately 90% and NPV of approximately 95% are used in an attempt to avoid liver biopsy in a theoretical population of 1000 patients with CHC. The percentage of that population in whom the use of markers would generate a result meeting these criteria for accuracy, and the proportion of biopsies correctly and incorrectly avoided are presented. For example, in the study by Wai , 51% of people had test results at these thresholds and would be able to avoid biopsies [102]. However this would mean that of these 7% would be false negatives and would be cases with significant fibrosis that were not detected by the markers.

Figure 2-1: Performance of surrogate markers in theoretical cohort of 1000 patients in distinguishing F0/1 versus F2/3/4



Results from studies differentiating (F0/F1/F2/F3 vs F4)

In studies that reported results for F0, 1, 2, 3 vs F4 (ie no cirrhosis versus cirrhosis) all of the surrogate markers performed at a higher level, with the AUC and sensitivity and specificity being greater at all thresholds (Table 2.4)

2.3 Discussion

14 primary studies were identified evaluating the diagnostic performance of serum markers of liver fibrosis in CHC, using 10 different combinations of serum markers. Whilst all studies recruited patients from specialist clinics, there was variation between studies in population characteristics, prevalence of severe fibrosis, and methods of test validation. Most studies reported AUC in differentiating mild /no fibrosis and significant fibrosis with the median value in validation populations being 0.77. These values, in addition to corresponding LR and DOR, are not commensurate with high diagnostic accuracy. All tests showed improved performance in differentiating cirrhosis/no cirrhosis with median AUC in validation sets of 0.87.

The findings of this systematic review can be applied in clinical practice to avoid biopsy using test threshold levels at illustrative predictive values identifying the presence or absence of significant fibrosis. At PPV 90% and NPV 95% tests were applicable to 35% of the study population. This figure can be increased by relaxing the probability of making correct assignment. This method of reporting test performance may be useful and help in the critical assessment of evidence by clinicians before using these markers in their practice. There is no consensus on what predictive values are acceptable to clinicians when assessing liver fibrosis. A PPV of 95 % and PPV 90 % were chosen as conservative thresholds following discussions with local clinicians. This issue of which thresholds should be chosen for clinical utility will be further explored in chapter 4.

Heterogeneity between studies may be attributable to population differences e.g. alcohol consumption, prevalence of severe fibrosis or tests used. The prevalence of significant fibrosis (F2, F3, F4) varied between studies from 17-80% (median 40%). Predictive values of tests are affected by disease prevalence, leading to a lack of generalisability to individual practice where proportion of significant fibrosis differs from included studies. Therefore knowledge of the fibrosis prevalence is necessary to determine appropriateness of a test to individual clinical practice. It is possible some tests might perform better in low or high prevalence populations, for example, those with a high sensitivity across lower test scores, will perform best in low prevalence populations as the NPV

will be higher and the test is applicable to a significant part of the study population; the converse would apply in high prevalence populations.

Most studies presented AUC as a measure of test performance, with good values being as close to 1.0 as possible. The median AUC in validation cohorts was 0.77, suggesting that few tests are excellent. However, AUC has limitations and may not be the best way to present test performance [113;114]. Whilst the panels included in this review almost all performed with good sensitivity and specificity at highest and lowest thresholds, the AUC does not adequately reflect the test performance at other thresholds where sensitivity and specificity are considerably lower. A misleadingly high value for the AUC may thus disguise the true diagnostic performance across all thresholds. Other diagnostic test evaluations have selected LR and DOR as better ways of evaluating and comparing tests and these measures may be more discriminating. A LR describes how many times more likely a person with the disease will receive a particular test result than a person without the disease. The median LR for panel serum markers was 8.2, with the majority falling outside the conventionally accepted "good" test range ($\geq$10). Some authors have suggested that the DOR is used [115].This describes the ratio of the odds of a positive test result in a patient with disease, compared to a patient without the disease (+ LR/-LR). A DOR of 1 suggests a test providing no diagnostic evidence, and a reasonable test may have DOR >30. DORs have been calculated for those studies that presented sufficient data, with the median DOR being 9 (range 5-27). DORs are not easy to apply in clinical practice, but are useful in comparisons such as when combining results in a systematic review and are reasonably constant regardless of the diagnostic threshold. The ability to derive a DOR is dependent upon the presentation of sufficient data to construct a 2x2 table.

Generally, methods of evaluating the quality of diagnostic tests are not as refined as those for therapy trials, with reporting of test evaluations similarly lagging behind [116]. An algorithm for reporting diagnostic tests, similar to the Consort statement for treatment trials, has been published- Standards for Reporting of Diagnostic Accuracy (STARD), and is gaining wider acceptance. This will contribute to the improvement of the quality of both the conduct and reporting of diagnostic evaluations [117;118]. QUADAS was applied to assess quality and found most studies used blinded

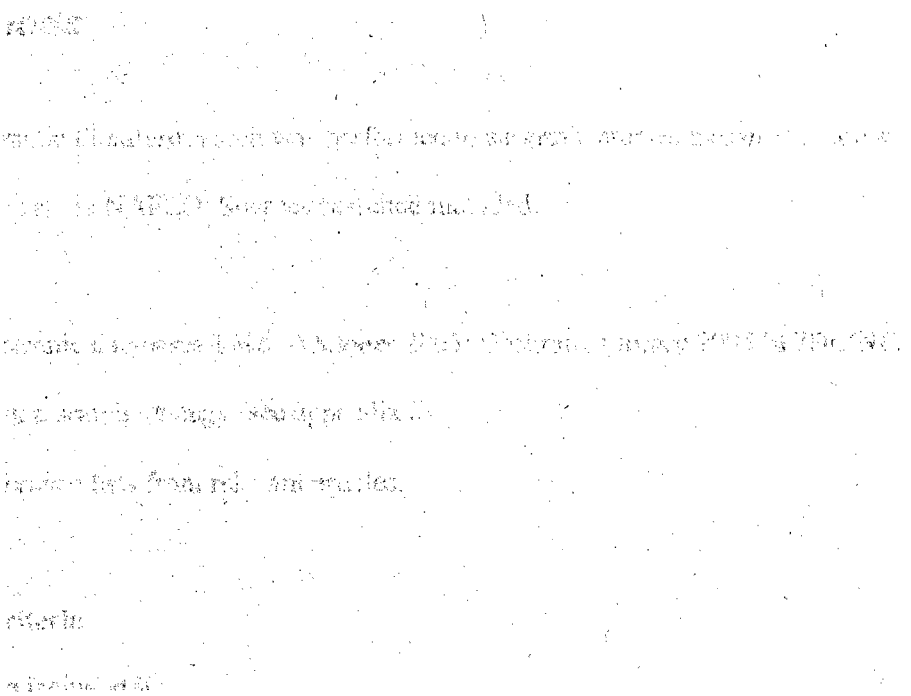
outcome assessment, were explicit about patient selection and exclusions, and used an accepted reference standard, all of which have been cited as the most important criteria that impact on study quality [119]. However, sources of potential bias were identified including the incomplete reporting of data (e.g. co-morbidity, alcohol consumption), and sensitivities and specificities at all thresholds. Some studies used the same cohort both to derive and evaluate the performance of the markers or an internal validation cohort, (where patients were recruited from a group similar to the training cohort) both of which limit generalisability of the findings. A more rigorous methodology would be to derive the panel in one cohort and validate in several external populations or (most preferably) a reference population in which different tests could be tested and directly compared using a standardised reference test.

Only studies with greater than thirty subjects were included in this systematic review. This is a potential limitation but there were a number of reasons for doing this. Firstly, smaller studies tend to be of lower methodological quality and their inclusion increases heterogeneity [120]. Secondly and perhaps more importantly, in order to calculate statistics reflecting diagnostic accuracy, it is important not to have empty cells in the 2 X 2 tables and this is much more likely in studies with small numbers. Performing a meta-analysis by combining different panel tests is difficult because of the issues of heterogeneity, as discussed above. Multiple studies on the same test, eg Fibrotest, could be potentially combined using Summary Receiver Operator Curves (SROC), to give an overall diagnostic summary statistic. The major caveat of this analysis is the limitations that are applicable to the simple AUC would still apply to the SROC statistic.

A fundamental methodological limitation in assessing non-invasive markers is the use of liver biopsy as a reference standard, and this may contribute to the moderate performance of these tests. In addition to the sampling error and observer variability raised in the introduction, there are difficulties in obtaining an adequate sample –some experts have suggested that 25mm in length, others that 11 portal tracts is optimum [87;121]. Data on the discordant results between histology and one panel of markers has been explored with attribution of discordance to biopsy failure in 18 % cases, failure of markers in 2.4% and non-attributable in 8.2% cases[122] . The authors concluded that

in many cases of differences it is the shortcomings of the biopsy that are responsible and this leads to an underestimation of the diagnostic performance of the serum markers.

The lack of a universal scoring system of fibrosis adds to the difficulties in comparison between studies. Whichever score is used, the histological staging of liver fibrosis on biopsy is artificially represented as a quantitative categorical variable with a linear quantum progression in severity from 0 to 4 or 6. This does not accurately reflect the dynamic biological process of fibrosis and constrains the serum marker test performances that are capable of generating continuous variables. Fibrosis progression is likely to be non-linear, and there is not equal temporal progression between sequential stages. It is therefore important to consider how to improve the reference standard in liver disease, or the use of a different reference standard, such as clinical outcomes (mortality or serious morbidity). The expense of these diagnostic trials and length of time required to reach such clinical end-points are major disadvantages.



Chapter 3 : Systematic review of serum markers in assessing fibrosis in Non-alcoholic fatty liver disease.

Background

Non-invasive markers of liver fibrosis have been most extensively studied in the context of hepatitis C. There has been considerable interest in extending this work into the field of NAFLD because of awareness of increasing prevalence of disease. The presumptive diagnosis of NAFLD is rapidly becoming the commonest cause for referral to hepatology out-patients clinics. Stratifying these patients for existing therapeutic intervention ,such as weight reduction, and potential pharmacological treatments via randomised clinical trials is dependent on making an assessment of the underlying severity of disease, in particular fibrosis. Moreover, recent long term studies suggest that the development of fibrosis within NAFLD has an important prognostic significance [123;124]. A systematic review was performed to assess the performance of non-invasive markers to assess liver fibrosis in NAFLD.

3.2 Methods

A systematic literature search was performed to ascertain studies measuring fibrosis by non-invasive markers in NAFLD. Sources searched included:

- Electronic databases 1996 – October 2005: Cochrane Library 2005 MEDLINE, EMBASE using a search strategy (see appendix 5).
- Reference lists from relevant articles.

Inclusion criteria

Studies were included if:

- they were systematic reviews, meta-analyses or studies of diagnostic tests
- they used liver biopsy as a reference standard.

- included >30 participants (as smaller studies will be underpowered to produce precise estimates of test performance).
- alcohol consumption of subjects was stated.
- reasonable attempt was made to exclude patients with other causes of liver disease eg alcohol and viral infections.

Studies identified by the search strategy were assessed for inclusion by two reviewers (NG and JP).

Exclusion criteria

Studies were excluded if;

- data on fibrosis stage(s) was not extractable
- data were presented only in abstract form.

Data extraction strategy

Data extraction was undertaken by one reviewer (NG) and checked by a second reviewer (JP) with any disagreements being resolved through discussion. A third reviewer was consulted (PR) to resolve persisting issues. Information collected included patient demographics, test assay details, background prevalence of fibrosis severity, risk factors, histological parameters, statistical methods used, and test performance characteristics. Where data was available 2 x 2 contingency were constructed to determine diagnostic accuracy statistics (eg sensitivity, specificity and predictive values) or odds ratios presented as a measure of association.

Quality assessment strategy

The quality of included studies was assessed using a modified quality assessment of diagnostic accuracy studies (QADAS) tool (Appendix 3).

3.3 Results

The electronic search yielded 1,781 abstracts which were read in full. 47 full papers were retrieved of which 18 were excluded leaving 29 studies in separate populations to be included in the review. Reasons for exclusion included: data on fibrosis stage was unavailable (n=10), less than 30 participants (n=5), test was not a panel marker (n=3).

The demographics of patients included in the final analysis are shown in table 3.1. The prevalence of severe fibrosis (grade 3-4) ranged from 9 % to 43 % with a median of 22.5%. The range of mean BMI in the studies was 26 to 60 (median 31); 5 studies recruited from patients undergoing bariatric surgery. The cut-off for alcohol consumption varied amongst studies but the majority excluded patients consuming > 200 g/week. Only 7 studies included details of length of biopsy specimen or number of portal tracts.

Three studies produced a diagnostic algorithm in association with specificities, sensitivities, predictive values and/or area under the receiving operator curve statistics (AUROC). The remaining studies investigated the association of individual variables with severe fibrosis vs. moderate fibrosis (17 studies), moderate fibrosis vs. mild fibrosis (4 studies), any fibrosis vs. no fibrosis (7 studies) and no fibrosis vs moderate fibrosis (1 study). Table 3.2 shows the variables positively associated with fibrosis.

Table 3-1: Characteristics of studies included in NAFLD review

Study Author Year of publication Date of Study Country	Total No. Patients	Patient selection	Prevalence of : Steatosis (S) Inflammation (I) Fibrosis (F)	Age Mean (median)	% male	BMI Mean (md)	Etoh	% diabetes	Liver biopsy Score Length (L) Portal tract (PT) Observers (O)	Non-invasive variables
Angulo[125] Hepatology 1999 USA	144	NAFLD on biopsy and persistently abnormal LFTs for more than 3 months. Prospective and retrospective recruitment	73 % grade 2-3 (S) 27 % significant fibrosis (F3/4)	(50.5)	33	31.2	<40 g/week	28 % diabetes	Modified Brunt L (n/s) PT (n/s) O (n/s)	Age AST/ ALT >1 ALT Albumin Transferrin saturation Diabetes
Rosenberg [110] Gastro 2004 Europe	61	NAFLD on biopsy and abnormal LFTs for 6 months. Prospective recruitment	27 % significant fibrosis (F3/4)	44	63	n/s	n/s	n/s	Sheuer L (>12 mm) PT (>5) O (3)	Age HA P IIINP TIMP-1
Sakugawa[126] World J Gastro 2005 Japan	112	NAFLD on biopsy	63 % NASH 43 % significant fibrosis (F3/4)	51	32	29	<30 g/d	30 % diabetes	Modified Brunt L (n/s) PT (n/s) O (2)	Female Platelets Albumin GGT, HA AST/ALT

Albano[127] Gut 2005 UK	167 (NAFLD) 59 (controls)	NAFLD on biopsy. Case controlled: NAFLD vs controls Prospective consecutive recruitment	44 % NASH 17 % significant fibrosis (F3/4)	55	61	35	<20g/d	29 % diabetes	Modified Brunt L (n/s) PT (n/s) O (1)	Age AST/ALT >1 Diabetes Malondialdehyde (MDA)
Mofrad[128] Hepatology 2003 USA	51	NAFLD on biopsy with normal ALT	72 % grade 2-3 (S) 36 % severe fibrosis (F3/4)	53	31	29	<20 g/d	57 % diabetes	Modified Brunt L (n/s) PT (n/s) O (1)	Diabetes
Shimada[129] Hepatology Res 2002 Japan	81	NASH on biopsy Prospective recruitment	82 % grade 2/3 (S) 100 % NASH 28 % severe fibrosis (F3/4)	(54)	49	(26)	<20 g/week	31 % diabetes	Brunt L (n/s) PT (n/s) O (1)	Age Platelet count, AST/ALT >1, Albumin, Bilirubin, ferritin, platelets, IgA, PT, type IV collagen, raised lipids
Dixon[130] Gastro 2001 Australia	105	Patients undergoing laparoscopic banding with BMI>35. Prospective consecutive recruitment	25 % NASH. 10 % severe fibrosis (F3/4)	41	21	47	<200 g/week	18 % diabetes	Brunt L (n/s) PT (>6) O (1)	Male Diabetes Hypertension ALT C peptide

Beymer[131] Archives of Surgery 2003 USA	48	BMI >35 undergoing gastric bypass surgery and liver biopsy Prospective consecutive recruitment	64 % grade 2/3 (S) 33 % NASH 12 % severe fibrosis (F3/4)	42	31	60	<20 g/mth	19 % diabetes	Ishak L (n/s) PT (n/s) O (1)	Diabetes
Bugianesi[132] Hepatology 2004 Italy	167	Raised transaminases (>6 months) and bright liver on U/S and NAFLD on biopsy. Prospective recruitment	47 % grade 2/3 (S) 21 % severe fibrosis (F3/4)	41	83	28	<20 g/d	8 % diabetes	Modified Brunt L (n/s) PT (n/s) O (n/s)	Age, female, BMI, AST/ALT Ferritin OGIS, 1/QUICKI HOMA-IR
Dixon[133] J Hep 2003 Australia	105	Patients with BMI >35 undergoing laparoscopic banding and liver biopsy Prospective recruitment	34 % NASH 14 % severe fibrosis (F 3/4)	42	26	>35	<200 g/week	n/s	Brunt L (n/s) PT (>6) O (1)	ALT HOMA IR Polymorphis-ms in transforming growth (TGF) and angio-tensinogen (AT)
Hui[134] Hepatology 2004	109 (NAFLD)	Patients referred with abnormal LFTS or hepatic steatosis on U/S and NAFLD on	50 % grade 2/3 (S) 73 % NASH	48	63	30	<40 g/week	32 % diabetes in NAFLD group	Brunt L (n/s) PT (n/s)	Age HOMA-IR

Australia	82 (controls)	biopsy. Controls matched by age and BMI. Case controlled and prospective	28 % severe fibrosis (F3/4)						O (1)	
Guidorizzi[135] Eur J Gastro/Hep 2005 Brazil	64	Patients with NAFLD on biopsy. Prospective recruitment	84 % NASH 11 % severe fibrosis (F3/4)	45	78	28	<20 g/day	11 % diabetes	Brunt L (n/s) PT (n/s) O (1)	HOMA-IR
Suzuki[136] Liver Int 2005 USA	79	Patients with abnormal LFTs for three months and NAFLD on liver biopsy Prospective and consecutive recruitment	25 % severe fibrosis (F3/4)	46	38	33	<40 g/week	n/s	Brunt L (>15 mm) PT (n/s) O (1)	Age Serum albumin Platelet count Fasting blood glucose Hyaluronic acid Clinical diagnostic score
Angulo[137] J Hep 2004 USA	88	Patients with abnormal LFTS, NAFLD on biopsy and participants in previous trials.	77 % grade 2-3 (S) 83 % NASH 22 % severe fibrosis (F3/4)	45	35	33	<140 g/week	19 % diabetes	Brunt L (>15 mm) PT (n/s)	Age Female BMI Diabetes Leptin

		Retrospective recruitment							O (1)	QUICKI HOMA IR
Marchesini[138] Hepatology 2003 Italy	163	Patients with abnormal LFTS for three months and NAFLD on liver biopsy. Prospective consecutive recruitment	74 % NASH 21 % severe fibrosis (F3/4)	40	88	28	<140 g/week		Brunt L (n/s) PT (n/s) O (n/s)	Metabolic syndrome
Hashimoto[139] Hep Research 2005 Japan	247	Patients with NAFLD on liver biopsy Prospective recruitment	36 % severe fibrosis (F3/4)	(53)	53	67 % with BMI >28	<100 g/week	33 % diabetes	Local score	Age Sex AST/ALT Albumin, Platelets Diabetes, hyaluronic acid and type IV collagen.
Ong[140] Obesity Surg 2005 USA (24)	212	Patients undergoing bariatric surgery with BMI >40 and obesity related complications. Prospective recruitment	24 % NASH 8 % advanced fibrosis	42	20	48	<10 g/day	24 % diabetes	Local score L (n/s) PT (n/s) O (1)	Waist to hip ratio (WHR) AST ALT Diabetes HT
Ledinghen[141] Eur J	67	Chronically elevated ALT for six months and liver biopsy	40 % NASH 31 % F2/3/4 fibrosis	47	67	26	<40 g/day	n/s	Metavir L (n/s)	BMI AST ALT

Gastro/Hep 2004		Retrospective recruitment							PT (n/s) O (1)	Ferritin
Ratziu[142] Gastro 2000 France	93	BMI >25, abnormal LFTS and NASH on liver biopsy. Retrospective consecutive recruitment	30 % F2/3/4 fibrosis	49	34	29	30 g/d	16 % diabetes	Metavir L (n/s) PT (n/s) O (1)	Age BMI ALT Diabetes Triglycerides
Sorrentino[143] J Hep 2004 Italy	80	Undergoing liver biopsy for operative procedure(gall stones, large bowel or gastric cancer) + metabolic syndrome + high grade obesity + normal LFTS Prospective recruitment	53 % grade 2/3 (S) 73 % NASH 23 % severe fibrosis (F3/4)	58	38	39	<30 g/day	45 % diabetes	Brunt L (>8 mm) PT (n/s) O (2)	Female BMI >45 Duration of obesity Metabolic syndrome
Crespo[144] Obesity Surgery 2001 Spain	181	Patients undergoing bariatric surgery and liver biopsy Prospective recruitment	72 % grade 2/3 (S) 23 % F2/3/4 fibrosis	n/s	16	47	<30 g/d	n/s	Modified Metavir L (n/s) PT (n/s) O (1)	Age at liver biopsy Elevated blood sugar level
Fierbinteanu-	80	Abnormal LFTS and	26 % NASH	51	25	32	<200	n/s	Local score	Age

Braticevici[145] Rom J Intern 2002 Romania		fatty liver on U/S and undergoing liver biopsy Retrospective recruitment					g/week		L (n/s) PT (n/s) O (1)	BMI >30 ALT >3 N Ferritin Triglycerides (TG) MDA Glutathione (GSH)
Loguerico[146] Dig Liver Dis 2004 Italy	305	Abnormal ALT for 12 months and NAFLD on liver biopsy Prospective recruitment	68 % grade2/3 (S) Mod/severe pericellular fibrosis	n/a	82	70 % were >25	<20 g/d	n/s	Local score L (n/s) PT (n/s) O (3)	Ferritin HOMA IR
Santos[147] Brazilian J Med 2005 Brazil	30	BMI >25 + U/S diagnosis of steatosis + raised LFTs and undergoing liver biopsy Prospective recruitment	Fibrosis present in 37 %	45	60	31	<20 g/day	23 % diabetes	Modified Brunt L (n/s) PT (n/s) O (n/s)	AST Laminin HA Collagen IV
Yesilova[148] Am J Gastro 2005 Turkey	51 (NAFLD) 30 (controls)	Raised LFTS for six months and NAFLD on liver biopsy Prospective recruitment	60 % grade2/3 (S) 88 % NASH 10 % severe fibrosis (F3/4)	36	100	28	<20 g/day	0 % diabetes	Brunt L (n/s) PT (n/s) O (n/s)	HOMA-IR Co enzyme Q10 Copper zinc oxide dismutase
Koruk[149]	36	Steatosis on U/S,	67 % Grade2/3	44	75	(29)	absent	20 %	Modified	Triglycerides

J Clin Gastroenterol 2003	(NASH) 32 (controls)	abnormal LFTs for three months and NASH on liver biopsy	(S) 100 % NASH 0 % severe fibrosis (F3/4)					diabetes	Brunt L (n/s) PT (n/s) O (n/s)	LDL cholesterol Apoprotein A1 (Apo A1)
Hartleb[150] Gastro Polska 2005	47	Patients with NAFLD on liver biopsy and ALT > 1.5 ULN Retrospective study	50 % Grade 2/3 (S) 65 % NASH 20 % some fibrosis	45	57	29	<120 g/week	13 % diabetes	Local L (n/s) PT (>5) O (2)	Age BMI Diabetes Hypertension
Chitturi[151] Hepatology 2002 Australia	94	NASH Prospective and retrospective	70 % Grade 2/3 (S) 45 % significant fibrosis (F3/4)	51	57	31	<20 g/d	47 % diabetes	Modified Brunt L (n/s) PT (n/s) O (1)	None
Brunt[152] Human Path 2004 USA	30	Subjects in NASH treatment trial. Retrospective	43 % grade1-4 fibrosis	45	46	34	<20 g/day	25 % diabetes	Brunt and Metavir L (n/s) PT (n/s) O (1)	AST/ALT ratio Albumin

Table 3-2: Variables associated with fibrosis in NAFLD

Category	Variable
Sociodemographic and anthropometric	Age, Gender, BMI, waist to hip ratio (WHR)
Simple liver biochemistry and haematology	ALT, AST, AST/ALT ratio, platelets, bilirubin, ferritin, transferrin sat, albumin.
Features of metabolic syndrome or glucose sensitivity	Diabetes, Hypertension, Homeostatic insulin resistance (HOMA-IR), Oral glucose sensitivity index (OGIS), metabolic syndrome, raised triglycerides, Quantitiative insulin sensitivity check index (QUICKI), adiponectin, leptin, hyperlipidaemia
Fibrosis markers	Hyaluronic acid (HA), tissue inhibitor of metalloproteases 1 (TIMP 1), laminin, type IV collagen, aminoterminal peptide of procollagen III (PIIINP).
Miscellanous	Malondialdehyde, C peptide, polymorphisms of transforming growth factor and angiotensinogen, IgA, glutathione, arachidonic acid, oxidised cardiolipin, Co enzyme Q and copper oxide dismutase.

The presence or absence of the most common variables associated with fibrosis and figure 3.1 represents the association of these variables with severe fibrosis vs moderate or none.

The presentation of odds ratios (ORs) as a measure of association was variable across the studies. Some studies did not present any, others only presented univariate data, and a minority presented multivariate data but again in the latter there was variation in which factors were included in the final model. This is exemplified by the variable diabetes in distinguishing severe

fibrosis from moderate or no fibrosis, i.e. F3/4 versus F0/1/2 (figure 3.2). Ten studies suggested an association of diabetes with severe fibrosis (6 by UVA and 4 by MVA) however only half the studies published ORs for these associations. There was a wide confidence interval of ORs presented; in diabetes there was a range of point estimate ORs from 1.3 to 75. Furthermore, when the heterogeneous nature of the derivation populations are taken into consideration we did not feel meta-analysis of these data was appropriate.

The three studies producing a panel marker diagnostic test with AUCs and cut-offs with relevant specificities and sensitivities included the BAAT score, HA score and ELF score. Only one of these studies included a validation cohort and the number of patients in these studies is relatively small; two studies compared F3/4 vs F0/1/2 and the other compared F2/3/4 vs F0/1. The AUC ranged form 0.84 to 0.92, see table 3.3.

Table 3-3: Panel marker tests in NAFLD

TEST	COMPONENTS OF PANEL	FIBROSIS STAGE	TRAINING OR VALIDATION	NUMBER	AUC
HA score	Age >45, obesity, AST/ALT ratio >1, diabetes, Hyaluronic acid	F3/4 vs F0/1/2	Training	79	0.92
ELF score	Age, hyaluronic acid, TIMP-1, PIIINP	F3/4 vs F0/1/2	Validation	61	0.87
BAAT score	Age, BMI, ALT, serum triglycerides	F2/3/4 vs F0/1	Training	93	0.84

Figure 3-1: Variables associated with severe fibrosis

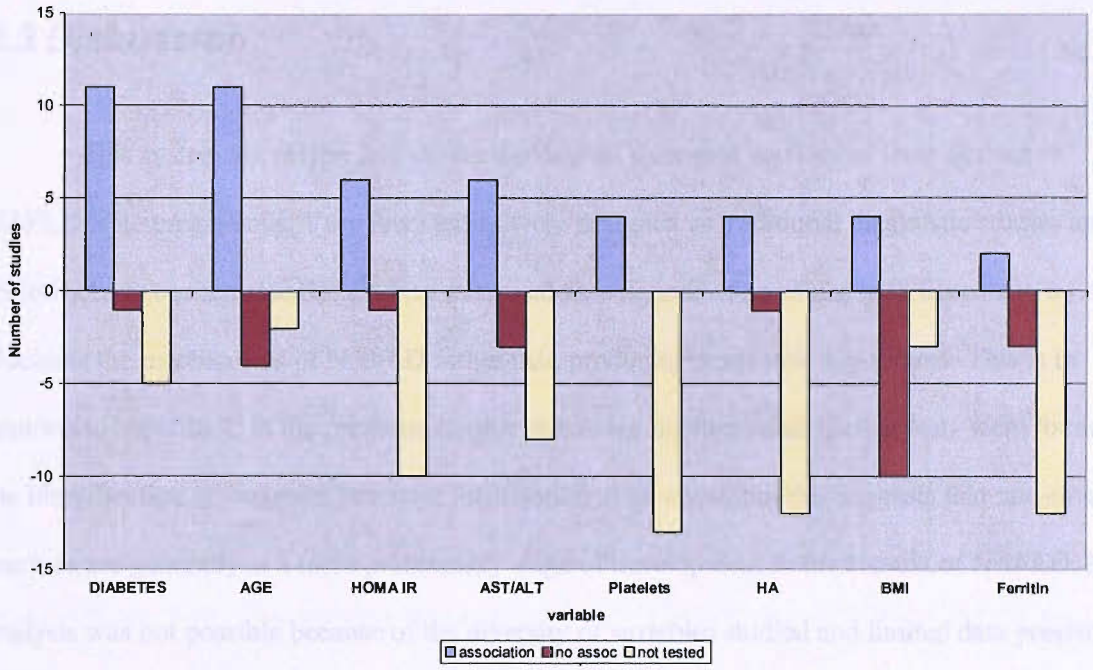
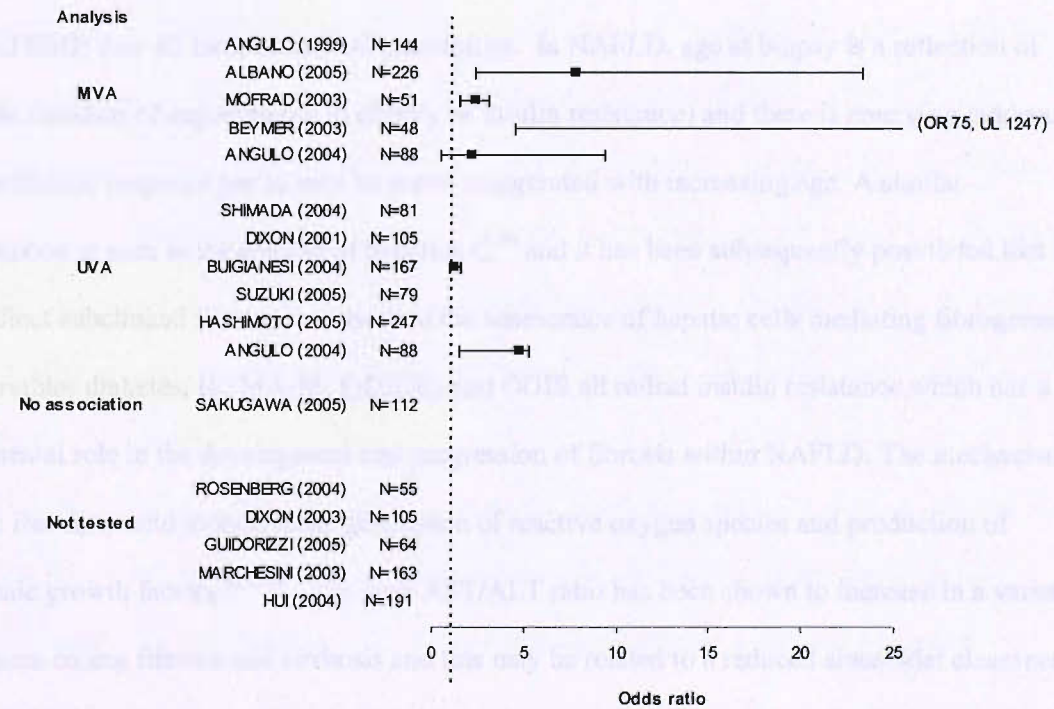


Figure 3-2: Forrest plot of strength of association of diabetes and severe fibrosis



footnote for 3.2: some studies only stated if diabetes was significant by MVA or UVA without stating OR.

3.3 Discussion

This systematic review has shown the data on surrogate markers of liver fibrosis in NAFLD is heterogeneous. Very few studies were designed as traditional diagnostic studies and the majority have concentrated on finding statistical associations of variables with fibrosis to try and elucidate the mechanisms of NAFLD rather than producing diagnostic algorithms. This is in contrast to hepatitis C in the previous chapter where ten distinct panel marker tests were found. As the identification of variables precedes formulation of an algorithm this suggests that non-invasive markers are generally at a more preliminary stage of development in the context of NAFLD. Meta-analysis was not possible because of the diversity of variables studied and limited data presented in the studies.

The variables most commonly positively associated with fibrosis are: presence of diabetes, increasing age, increased HOMA-IR, increased AST/ALT ratio, decreased platelets, hyaluronic acid and BMI; they all have biological plausibility. In NAFLD, age at biopsy is a reflection of probable duration of exposure (eg to obesity or insulin resistance) and there is emerging evidence that the fibrotic response per se may be more exaggerated with increasing age. A similar phenomenon is seen in the context of hepatitis C [30] and it has been subsequently postulated that this may reflect subclinical fibrosis or related to the senescence of hepatic cells mediating fibrogenesis. The variables diabetes, HOMA-IR, QUICKI and OGIS all reflect insulin resistance which has a fundamental role in the development and progression of fibrosis within NAFLD. The mechanisms include free fatty acid mobilisation, generation of reactive oxygen species and production of fibrogenic growth factors [153;154] . The high AST/ALT ratio has been shown to increase in a variety of diseases casing fibrosis and cirrhosis and this may be related to a reduced sinusoidal clearance of AST relative to ALT. Reduced platelets occur as a result of portal hypertension but also some chronic liver diseases may reduce the hormone thrombopoietin which stimulates platelet production. In NAFLD it appears to be a better indicator of severe fibrosis/cirrhosis rather than the

subtler stages of fibrosis. Finally hyaluronic acid may increase in fibrosis due to a mixture of increased collagen turnover and reduced hepatic clearance and this has been shown to increase in other aetiologies of liver fibrosis such as alcohol, hepatitis B and hepatitis C [155-157].

The three diagnostic tests that combined variables to produce a panel test resulted in AUCs ranging from 0.84 to 0.92. Results of independent validation of these algorithms will determine if they can be used across diverse populations. Furthermore, they all have reduced accuracy at mid-point thresholds and at extreme thresholds the cut-offs apply to a small proportion of population tested. Finally, two of the three panel tests used severe fibrosis (F3/4) as the diagnostic measure.

One of the reasons why the field of non-invasive markers may lag behind hepatitis C is because of uncertainty of which end point to measure i.e. simple steatosis, NASH , fibrosis or a combination of these histological states. Although recent studies suggest fibrosis has the greatest implication on prognosis there is clearly a need to identify precursor states of disease. This will be important for targeting patients in the future who will get maximal benefit from early therapeutic intervention. The field is divided into studies attempting to distinguish stages of fibrosis, fibrosis from NASH and NASH form simple steatosis. The variables separating these different entities are similar (unpublished observations) but it is likely that they will be required to be combined in differing algorithms to maximise diagnostic accuracy. An interesting observation is that within fibrosis, many of the studies in NAFLD have focussed on separating F0/1/2 from F3/4 and there does not seem to be the diversity of end-points compared to hepatitis C. Whether this is because of differing biology of disease or the stage of development of non-invasive tests remains to be determined.

A potential limitation of diagnostic reviews is publication bias. Indexing of such studies is problematic. A sensitive search strategy was used but it is likely some studies were missed especially those with non-significant results. Other limitations relate to the assessment of diagnostic accuracy. There is a clear balance between obtaining a diverse derivation population that mirrors clinical practice and in whom the diagnostic uncertainty exists (ie to limit so called spectrum bias) to increase generalisability of a test and feasibility of choosing a study population on whom one can obtain liver biopsies. The studies included in this review do vary in recruitment

methods and patient characteristics but there is a degree of selection bias partly due to the requirement of a liver biopsy as the reference test. For example five out of 27 studies included patients undergoing bariatric surgery. Patients in theses studies had a greater BMI and increased prevalence of severe fibrosis. Many studies only included patients with abnormal LFTs, reflecting clinical referral pathways to hepatology. Overall the effect may be to find associations at the severe end of spectrum of disease and or within a restricted range of exposure

Although, the studies appeared to perform well on the quality measure QUADAS (appendix 6) there were some general deficiencies. The reporting of size and quality of biopsy was poor. The variability of presentation of detailed statistical analysis such as ORs, with respective confidence intervals, makes it unclear how strong the associations are between variables and end-points. Results of multivariate analysis will depend on what other factors are put in the model and often this was not stated in the papers. For example, insulin sensitivity is clearly important in NAFLD but it is unclear if a combination of variables eg diabetes, HOMA-IR, QUICKI and OGIS is required because they are additive or if there is a summary measure.

Overall conclusions from systematic reviews in NAFLD and chronic hepatitis C

Both reviews provide an insight into the current literature for the non-invasive diagnosis of liver fibrosis in two of the major aetiologies of liver disease. The reviews highlighted that in the field of NAFLD non-invasive markers consist of single marker tests or variables. The number of true "diagnostic " tests that have been derived on a training population and tested on an independent validation population are relatively few compared to hepatitis C. The reasons for this are not entirely clear but there may be several explanations. The ascertainment of "index" cases of NAFLD is more difficult because of the lack of a clear diagnostic test. In CHC for example, the diagnosis is relatively clear cut; virus can be measured directly in the blood (using polymerase chain reaction method). In CHC there are less end-points of disease to measure, i.e. fibrosis and necroinflammation versus steatosis, steatohepatitis and fibrosis in NAFLD. Finally, there are clear pathways for the management of disease in CHC. This is not the case for NAFLD and currently there are no clear management algorithms for the different stages of disease.

The serum markers have high diagnostic accuracy at the extreme thresholds. The performance of the serum markers is limited by the use of an "imperfect" gold standard thus creating an artificial glass ceiling. The overall summary statistic in diagnostic tests of the AUC is useful in comparing different tests but measures such as the diagnostic odds ratios may be more important in dissecting individual tests. This is of particular relevance in the diagnosis of liver fibrosis where clinicians may only want to use the test at thresholds with high diagnostic accuracy because of the implication of missing disease or falsely assigning disease. The diagnostic end-points of serum markers eg mild vs moderate/severe disease may be sufficient but this will be dependent on the specific disease.

A number of questions arise from the previous chapters. Can the serum markers in their present form be used in clinical practice? Can existing algorithms be improved upon, and if so how? Can new markers be identified to increase diagnostic accuracy? The following chapters will attempt to address these questions.

Chapter 4 : Validating and exploring serum markers in NAFLD

Background

To further explore the role of serum panel markers in liver fibrosis non-alcoholic fatty liver disease (NAFLD) was chosen as the aetiology of disease. The major reason for choosing NAFLD was because of the finding in the systematic reviews that non-invasive markers were less developed in this area and many of the studies concentrated on severe fibrosis as an end point. Whilst this is undoubtedly important for prognosis, diagnosis of any fibrosis may be important at identifying individuals at earlier stage for active intervention; currently this may be only life style measures but in the future this is likely to involve pharmacological intervention.

The Original European Liver fibrosis (OELF) is a panel marker and consists of age, Tissue inhibitor of metalloproteinase 1(TIMP 1), hyaluronic acid (HA) and aminoterminal peptide of pro-collagen III (PIIINP) and was initially developed for a variety of liver aetiologies [110]. The OELF panel in the discovery study performed very well in the NAFLD subgroup. A simplified version of OELF, which omits age from the algorithm, the Enhanced Liver fibrosis panel (ELF) has subsequently been shown to have equal diagnostic performance in other liver diseases (Parkes et al, manuscript in preparation). The first aim was to validate the performance of OELF and ELF in an independent cohort of exclusively NAFLD patients.

The systematic review in NAFLD highlighted that "simple markers", i.e. solitary tests that are relatively cheap and are available in routine clinical practice, have a role in distinguishing severe fibrosis. The second aim was therefore to compare the performance of the ELF markers with simple markers in the same cohort and to test the hypothesis that a combination of markers would lead to an improvement in diagnostic performance. The final aim was to further explore the clinical utility model, developed in chapter 2, in the context of NAFLD and to see what impact this could have on current clinical practice.

4.1 Methods

Patients were recruited consecutively from two tertiary out-patient liver centres in the UK, Nottingham and Newcastle-upon-Tyne. The diagnosis of NAFLD was based on the following criteria: 1) elevated aminotransferases (AST and/or ALT); 2) appropriate exclusion of liver disease of other etiology including alcohol or drug induced liver disease, autoimmune or viral hepatitis, cholestatic or metabolic/genetic liver disease. These other liver diseases were excluded using specific clinical, biochemical, radiographic and /or histological criteria. All patients had a negative history of ethanol consumption of less than 140 g in women and 210 g in men. In the Newcastle center, alcohol levels in urine were measured randomly to rule out patients who abused alcohol. Patients included in this study were consecutive liver biopsies at the individual centers where the histology was consistent with NAFLD and serum samples were taken within three months of biopsy. The following clinical measurements were obtained: waist circumference, hip circumference and body mass index (BMI). Serum samples were obtained for routine liver chemistry (including alanine transaminase (ALT), gamma glutamyl transpeptidase, bilirubin, albumin, and alkaline phosphatase), full blood count, measures of insulin resistance (including fasting glucose, insulin and c peptide), ferritin, total cholesterol, HDL, LDL and triglycerides. Serum samples were analysed for levels of TIMP-1, HA and PIINP at an independent reference laboratory (iQqur Limited, Southampton, UK- details of SOP for assay in appendix 9). Results were entered into an established algorithm [110] and expressed as a Discriminant scores (DS).

Liver biopsy

Liver biopsies were assessed by two hepatopathologists, one at each centre, blind to the serum marker test results. Biopsies were graded for fibrosis using a five stage classification system for fibrosis that has recently been published by NIDDK [158]: stage 0= absence of fibrosis, stage 1 = perisinusoidal or portal, stage 2= perisinusoidal and portal/periportal, stage 3= septal or bridging fibrosis and stage 4= cirrhosis.

Statistical analysis

Discriminant scores were compared to histological staging of liver biopsies from corresponding patients and the sensitivity and specificity of the DS for detecting fibrosis was calculated. These results were then used to plot Receiver Operator Characteristic (ROC) curves and the Area Under the Curve (AUC) was calculated. Positive (PPV) and negative (NPV) predictive values for detecting different degrees of severity of fibrosis were also calculated.

Validation of OELF panel

The OELF panel was validated in the cohort using the original algorithm and an adjusted algorithm not containing age. Three end points were chosen for the evaluation of fibrosis i) any fibrosis (stage 0 versus 1/2/3/4) ii) moderate fibrosis (stage 0/1 versus 2/3/4) and iii) severe fibrosis (stage 0/12 versus 3/4). The modified ELF (i.e. OELF without age), called the Enhanced Liver Fibrosis (ELF) test, was used for the remaining analysis.

OELF algorithm

DS= -6.38 - (ln(age)*0.14) + (ln(HA)*0.616) + (ln(P3NP)*0.586) + (ln(TIMP1)*0.472).

ELF algorithm

DS = -7.412 + (ln(HA)*0.681) + (ln(P3NP)*0.775) + (ln(TIMP1)*0.494).

Comparison of the Enhanced Liver Fibrosis (ELF) panel with simple clinical and biochemical parameters

To compare the ELF panel to a panel of simple markers new algorithms were created using "simple" clinical and laboratory variables, listed above, using multivariate logistic regression. Forward logistic regression was employed, and variables with a p value less than 0.1 were selected for the final model. Separate panels were created for each of the separate end points; No fibrosis, moderate fibrosis and severe fibrosis. Finally, the ELF panel and simple markers were combined to see if diagnostic accuracy could be improved. The three algorithms i) ELF panel ii) simple panel

and iiii) combined panel (ELF + simple) were tested on the three end-points of fibrosis as stated in the previous section. ROC curves produced to compare diagnostic performance. All analyses were carried out using SPSS 14.

4.2 Results

Paired serum and histological data were available for 192 subjects. The baseline characteristics of these patients are shown in table 1. The demographic data were similar for the two populations; overall 64 % of subjects were male, the mean age in the study was 49 years and 63 % of subjects had evidence of the metabolic syndrome.

Table 4-1: Baseline patient characteristics in individual and combined cohorts

Category	Nottingham centre	Newcastle centre	Entire cohort
Number	88	104	192
Age (years)	50.4 +/- 11.5	47.3 +/- 11.1	48.7 +/- 12.5
Male subjects	65 %	63 %	64 %
BMI (kg/m^2)	30 +/- 4.5	34.4 +/- 5.9	32.4 +/- 5.7
Waist (cm)	104.5 +/- 12.5	111.2 +/- 12.7	107.8 +/- 13
Metabolic syndrome (yes)	66 %	60 %	63 %
Fasting Glucose (mmol/l)	6.0 +/- 1.7	6.5 +/- 3.3	6.3 +/- 2.7
Triglycerides (mmmol/l)	2.1 +/- 1.6	2.8 +/- 1.8	2.5 +/- 1.8
HDL (mmmol/l)	1.4 +/- 0.42	1.1 +/- 0.28	1.2 +/- 0.4
ALT (U/l)	76.1 +/- 48.9	78.4 +/- 64.6	77.3 +/- 57.8
GGT (U/l)	140 +/- 135	104 +/- 102	121 +/- 119.5
Albumin (g/l)	43.7 +/- 3.4	44.9 +/- 4.9	44.3 +/- 4.3
Fibrosis stage			
0	32 %	49 %	41 %
1	18 %	19 %	19 %
2	27 %	8 %	17 %
3	15 %	12%	13 %
4	8 %	12%	10 %

Footnote: Values in mean +/- standard deviation unless stated

Performance of the ELF panel

The OELF panel and ELF panel had a similar performance in the cohort in all three end-points when comparing AUC values and distribution of score (see figure 4.1). Therefore to simplify

the algorithm we omitted age and used the Enhanced Liver Fibrosis Panel for the remaining analysis. The ELF panel had an excellent performance in distinguishing severe fibrosis with an AUC of 0.90 (CI 0.84 to 0.96) and a threshold of 0.3576 was associated with a sensitivity of 80 %, specificity of 90 %, positive predictive value of 71 % and a negative predictive value of 94 %. In distinguishing moderate fibrosis the overall AUC was 0.82 (CI 0.75 to 0.89) and a threshold of -0.1068 was associated with a sensitivity of 70 %, specificity of 80 % , positive predictive value of 70 % and a negative predictive value of 80 %. In distinguishing no fibrosis the overall AUC was 0.76 (CI 0.69 to 0.83) and a threshold of -0.2070 was associated with a sensitivity of 61 %,, specificity of 80 % , positive predictive value of 81 % and a negative predictive value of 79 %. See table 4.2 for a full list of thresholds associated with the different end-points of fibrosis.

Figure 4-1: Box plots for OELF and ELF for discriminant score and fibrosis stage

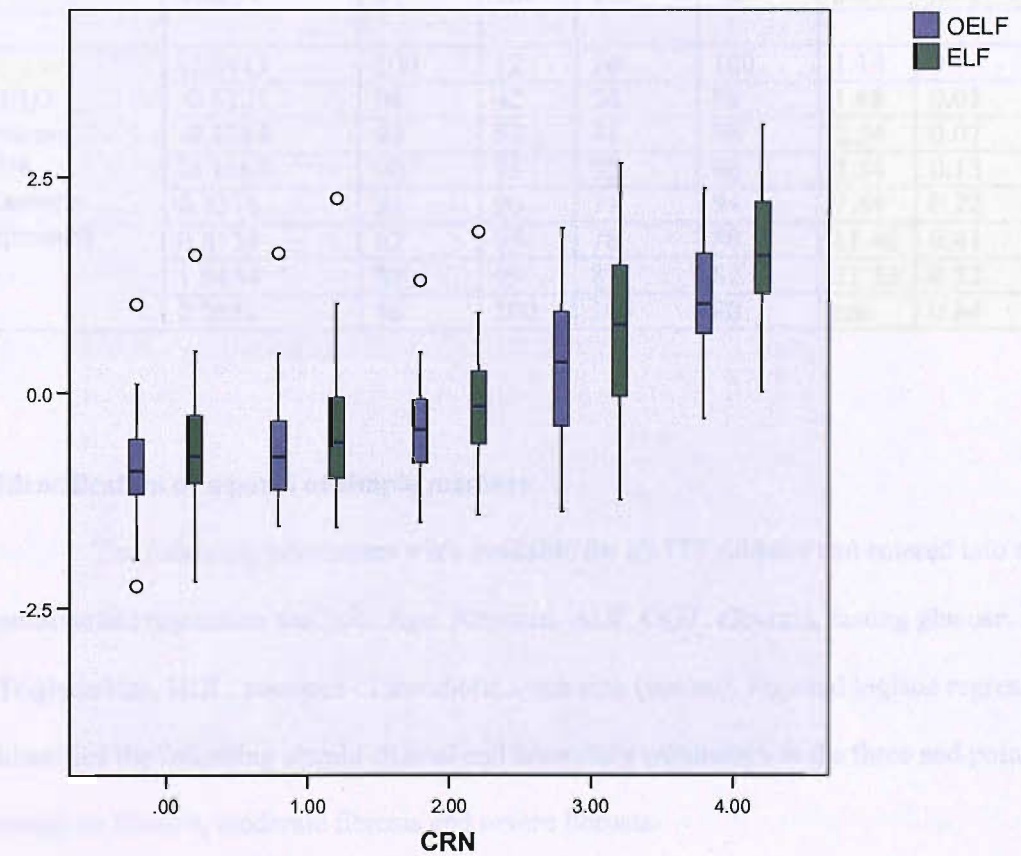


Table 4-2: Diagnostic performance of the Enhanced Liver Fibrosis Panel at different thresholds in the combined cohort

Stage of Fibrosis	Discriminant Score Threshold	Sens	Spec	PPV	NPV	LR +ve	LR -ve
0 versus 1/2/3/4 (any fibrosis)	-1.6533	100	4	60	100	1.04	0
	-1.2009	95	19	63	71	1.17	0.28
	-1.02813	90	30	64	66	1.23	0.36
	-0.6415	80	56	72	66	1.80	0.37
	-0.2070	61	80	81	79	3.01	0.49
	0.2112	49	90	86	53	4.47	0.61
	0.3272	43	95	93	54	8.67	0.59
	1.6454	13	100	100	45	n/a	0.87
0/1 versus 2/3/4 (moderate fibrosis)	-1.4217	100	7	42	100	1	0
	-1.0691	95	22	45	86	1.21	0.24
	-0.6746	90	50	54	88	1.78	0.21
	-0.3625	80	67	62	84	2.44	0.29
	-0.1068	70	80	70	80	3.51	0.37
	0.3145	56	90	78	75	5.35	0.49
	0.5734	45	95	85	72	8.71	0.57
	2.2859	9	100	100	62	n/a	0.91
0/1/2 versus 3/4 (severe fibrosis)	-1.2413	100	12	26	100	1.14	0
	-0.7121	98	42	34	98	1.68	0.05
	-0.4184	96	57	41	98	2.24	0.07
	-0.1068	90	75	52	96	3.54	0.15
	0.3576	80	90	71	94	7.84	0.22
	0.8139	62	95	78	89	11.40	0.41
	1.6454	29	99	87	82	21.32	0.72
	2.2858	16	100	100	80	n/a	0.84

Identification of a panel of simple markers

The following parameters were available for all 179 subjects and entered into the multivariate regression analysis: Age, Albumin, ALT, GGT, albumin, fasting glucose, BMI, Triglycerides, HDL, presence of metabolic syndrome (yes/no). Forward logistic regression identified the following simple clinical and laboratory parameters in the three end points of the study; no fibrosis, moderate fibrosis and severe fibrosis.

Simple panel for distinguishing no fibrosis

Score = 2.474 + 0.153 *glucose (mmol/l) + 0.01*ALT + 0.036*age (years) – 0.125 *alb (g/l)

Combined panel for distinguishing no fibrosis

Score = 2.678 + 1.281*ELF (discriminant score) + 0.156 *glucose(mmol/l) + 0.06*ALT – 0.076*alb (g/l)

Simple panel for distinguishing moderate fibrosis

Score = -1.082 - 0.951*metabolic syndrome (yes=0,no=1) + 0.08*age (years) + 0.0.61 *BMI (kg/m2) + 0.004 GGT – 0.121*alb (g/l)

Combined panel for distinguishing moderate fibrosis

Score = 4.012 + 1.562 *ELF (discriminant score) - 0.683*metabolic syndrome (yes=0, no=1) + 0.004 GGT – 0.099*alb (g/l)

Simple panel for distinguishing severe fibrosis

Score = -3.284 + 0.239 *glucose(mmol/l) + 0.146 *BMI (kg/m2) + 0.095*age (years) + 0.009*GGT – 2.733* HDL(mmol/l) - 0.160*alb (g/l)

Combined panel for distinguishing severe fibrosis

Score = -2.050 + 2.070*ELF (discriminant score) +0.265 *glucose(mmol/l) + 0.005*GGT – 1.595* HDL(mmol/l)

The performance of the simple panel is shown in table 4.3 in comparison to ELF and combining ELF with the simple markers and the individual ROC curves are shown in figures 4.2 to 4.4. The best algorithm is the combination of simple markers and ELF algorithm.

Table 4-3: Performance of ELF panel and simple markers panel in distinguishing different stages of fibrosis as measured by AUC values with standard error.

	0 versus 1/2/34 Any fibrosis	0/1 versus 2/3/4 Moderate fibrosis	0/1/2 versus 3 /4 Severe fibrosis
SIMPLE	0.74 +/- 0.03	0.80 +/- 0.03	0.88 +/- 0.03
ELF	0.77 +/- 0.03	0.82 +/- 0.03	0.91 +/- 0.03
COMBINED	0.81 +/- 0.03	0.85 +/- 0.03	0.93 +/- 0.02

Figure 4-2: ROC curves of simple panel, elf panel and combined panel in distinguishing no fibrosis

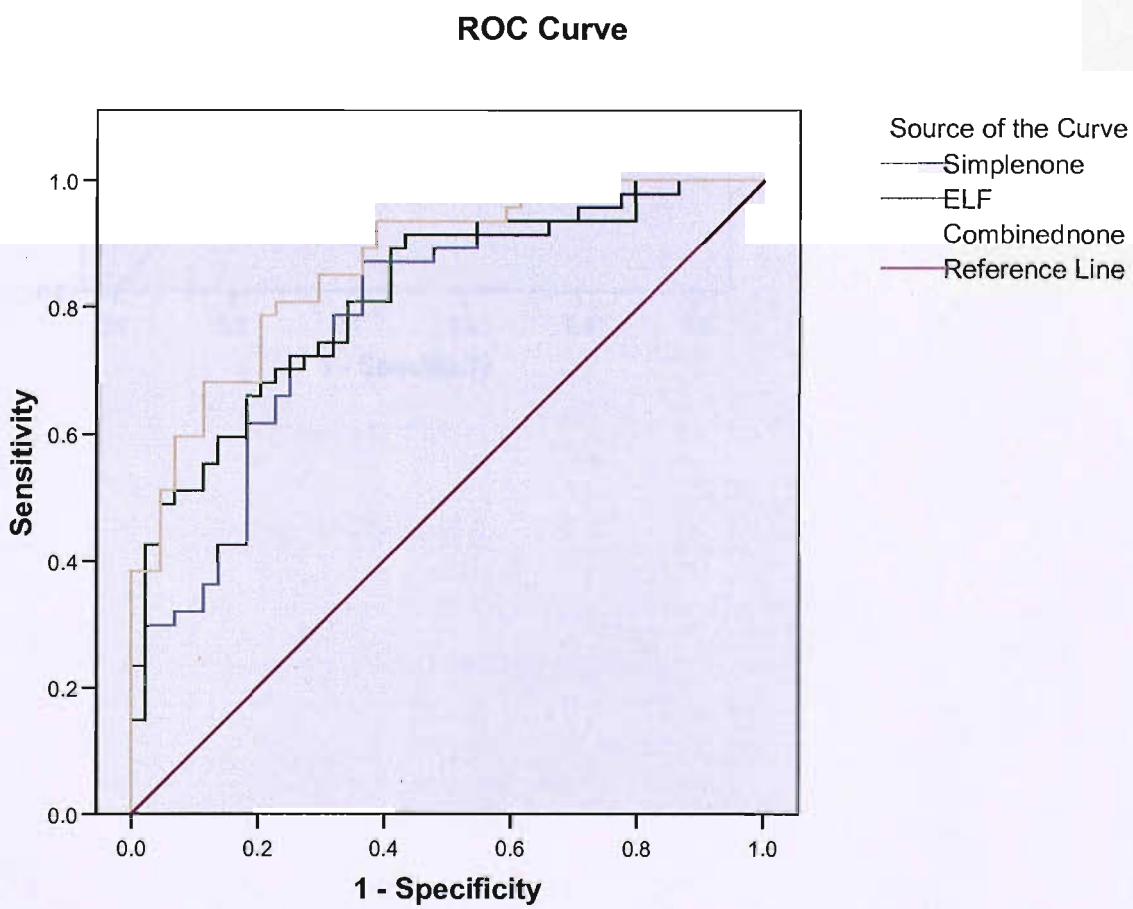


Figure 4-3: ROC curves of simple panel, elf panel and combined panel in distinguishing moderate fibrosis.

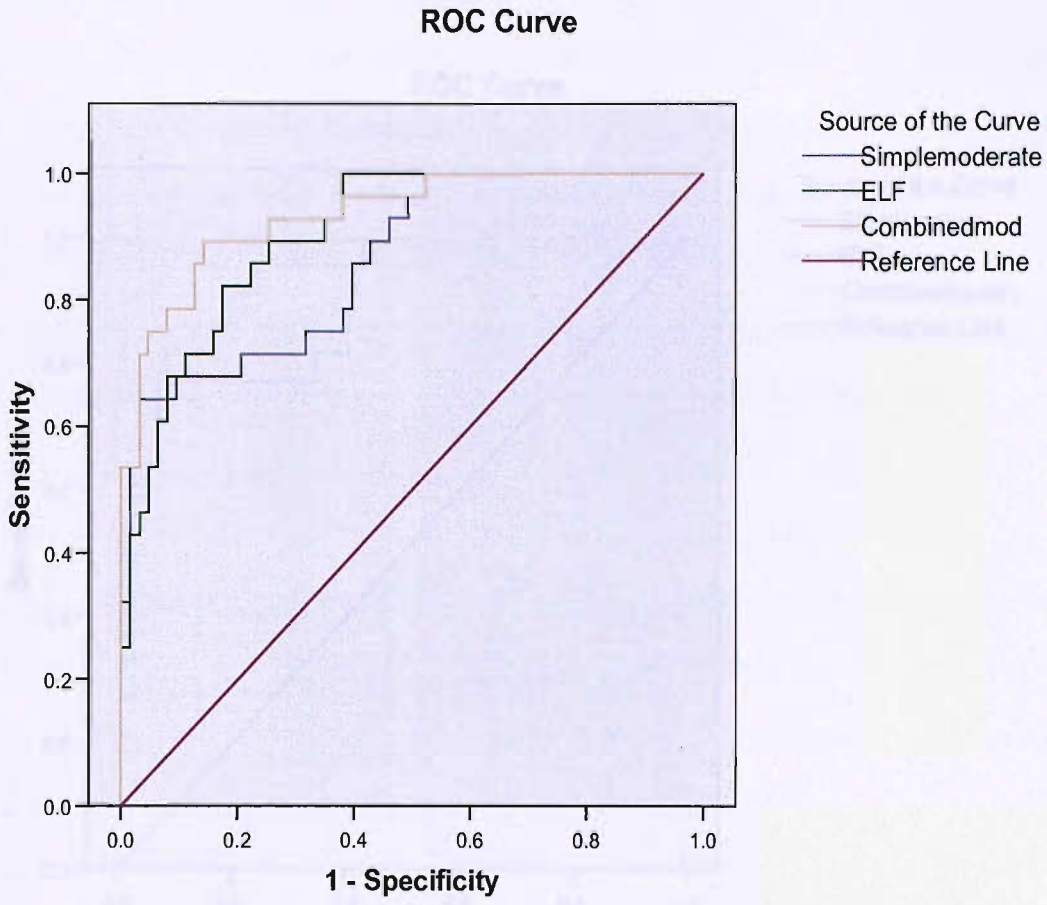
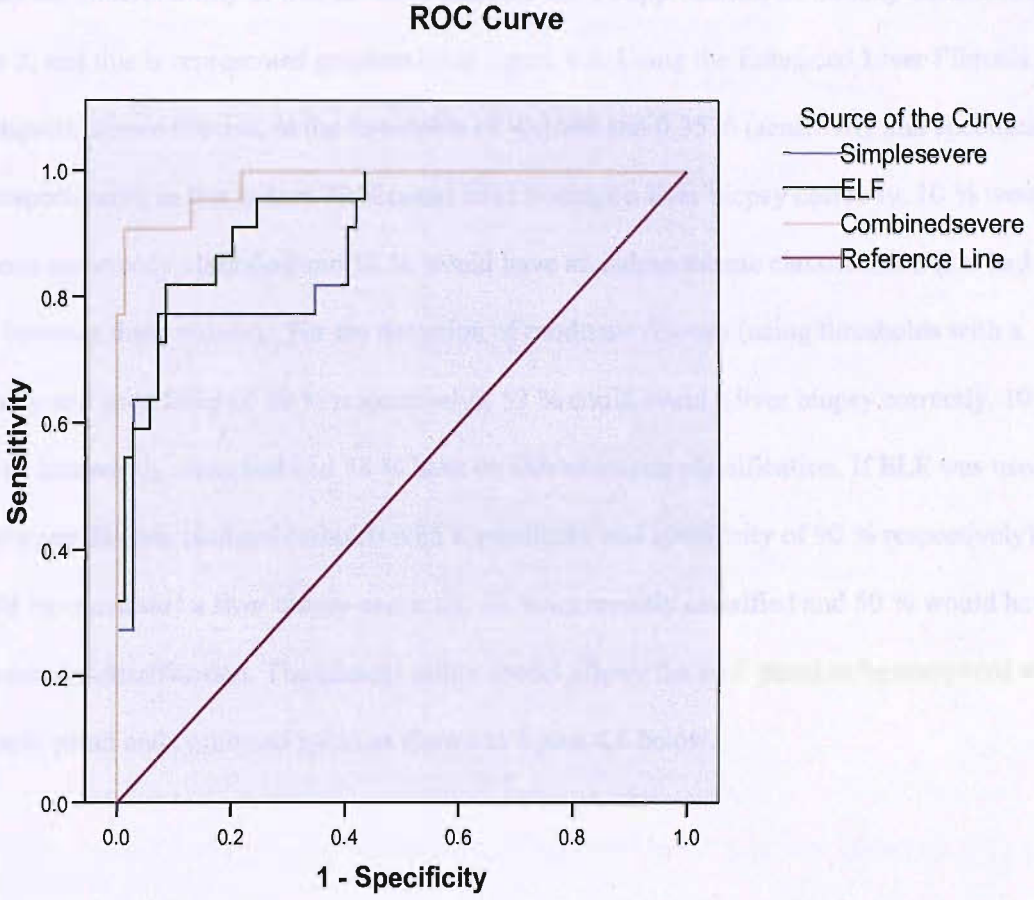


Figure 4-4: ROC curves of simple panel, elf panel and combined panel in distinguishing severe fibrosis



Clinical Utility of non-invasive markers in NAFLD

If thresholds are used to" rule in" fibrosis (upper threshold with high PPV and specificity) or "rule out" fibrosis (lower threshold with high NPV and sensitivity) with a high degree of accuracy the clinical utility of non-invasive markers can be appreciated, as initially described in chapter 2, and this is represented graphically in figure 4.5. Using the Enhanced Liver Fibrosis panel to distinguish severe fibrosis, at the thresholds of -0.1068 and 0.3576 (sensitivity and specificity of 90 % respectively), in this cohort 79 % could have avoided a liver biopsy correctly, 10 % would have been incorrectly classified and 11 % would have an indeterminate classification (i.e. had values between these values). For the detection of moderate fibrosis (using thresholds with a sensitivity and specificity of 90 % respectively), 52 % could avoid a liver biopsy correctly, 10 % would be incorrectly classified and 38 % have an indeterminate classification. If ELF was used to delineate any fibrosis (using thresholds with a sensitivity and specificity of 90 % respectively) 40 % could have avoided a liver biopsy correctly, 10 % incorrectly classified and 50 % would have an indeterminate classification. The clinical utility model allows the ELF panel to be compared with the simple panel and combined panel as shown in figure 4.6 below.

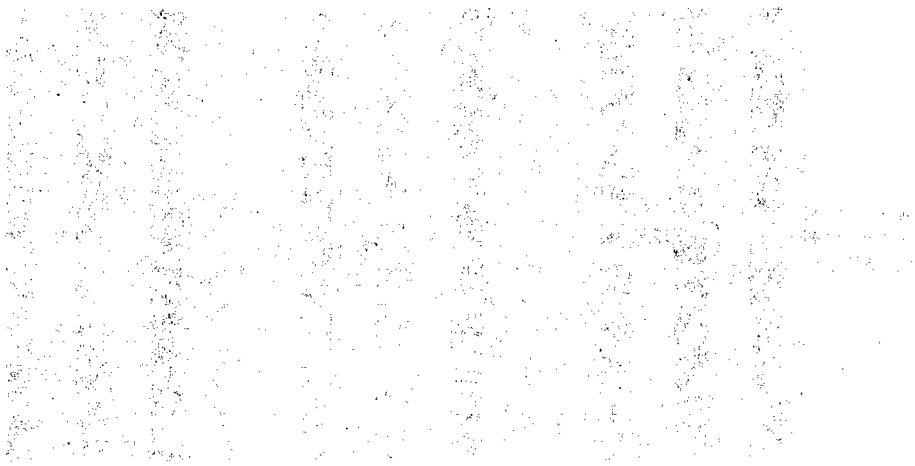
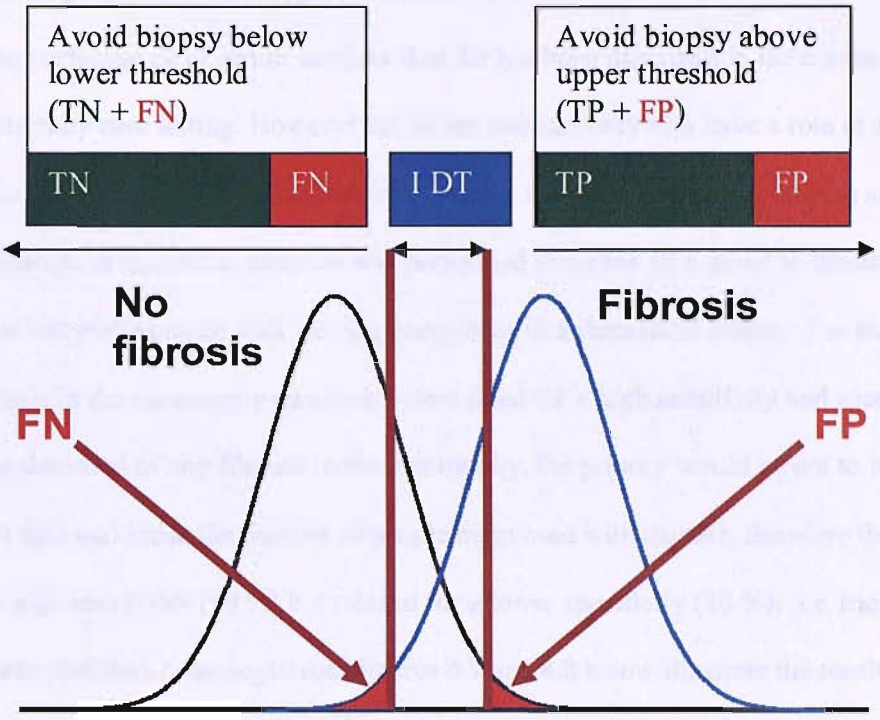
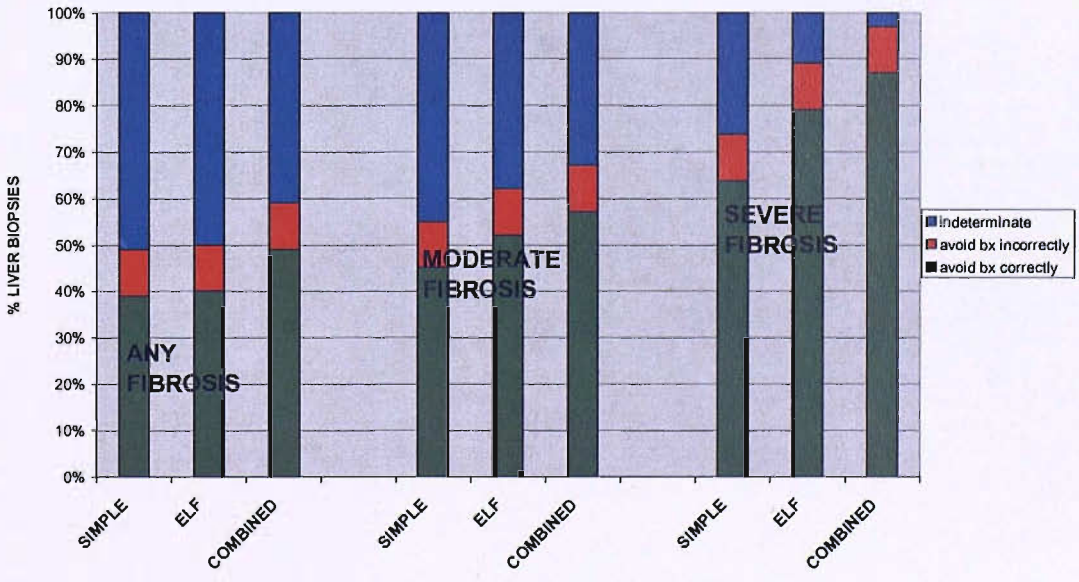


Figure 4-5: Methodology underlying clinical utility model



Key: 1) TN = True negatives 2) TP = True positives 3) FN= False negatives 4) FP= False positives 5) IDT= Indeterminate

Figure 4-6: Clinical Utility model of simple, ELF and combined panel in distinguishing different stages of fibrosis



The impact of prevalence on test performance

The performance of serum markers thus far has been described in the context of a secondary/tertiary care setting. However the serum markers may also have a role in a lower prevalence setting e.g. primary care. Predictive values will change with prevalence and thus affect test performance. A modelling exercise was performed using the ELF panel to illustrate the alteration in test performance with varying prevalence in a theoretical cohort. For the detection of severe fibrosis in the community thresholds were fixed for a high sensitivity and specificity (>90 %). For the detection of any fibrosis in the community, the priority would be not to miss any fibrosis but also maximise the number of people diagnosed with disease, therefore thresholds were fixed for a high sensitivity (90 %) but relaxed for a lower specificity (80 %); i.e. more false positives tolerated than false negatives. Figures 4.7 and 4.8 below illustrate the results of the modelling.

For the detection of severe fibrosis in primary care the model shows that the number of biopsies avoided can be increased to over 70 %, whilst keeping the number incorrectly avoided below 5 % when the prevalence of severe disease is reduced below 10 %. However, for the detection of any fibrosis, the number of biopsies correctly avoided reduces from 38 % to 21 %, when the prevalence of any disease falls to 30 %.

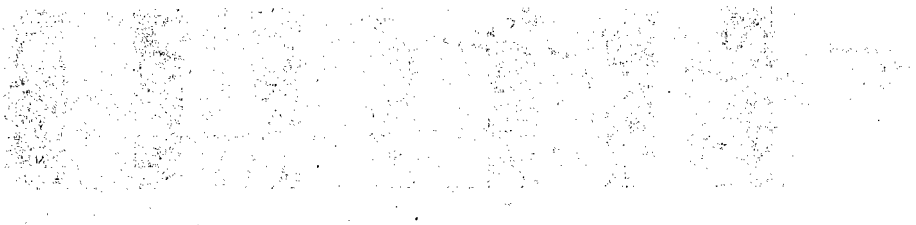


Figure 4-7: Clinical Utility Models showing effect of changing prevalence on diagnosis of severe fibrosis in a theoretical cohort.

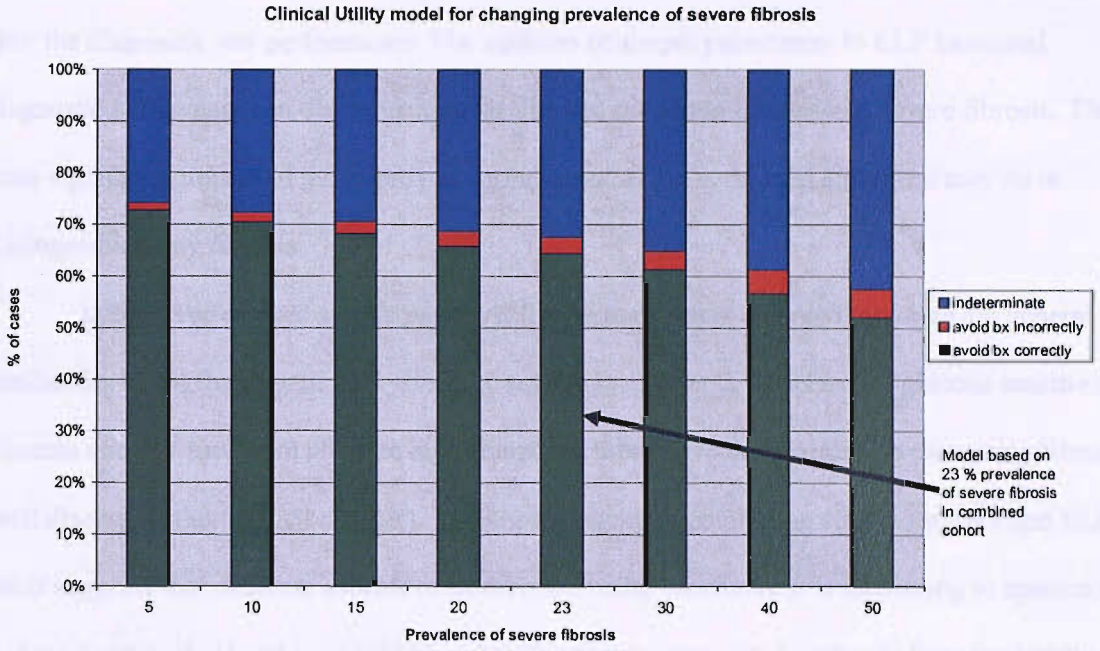
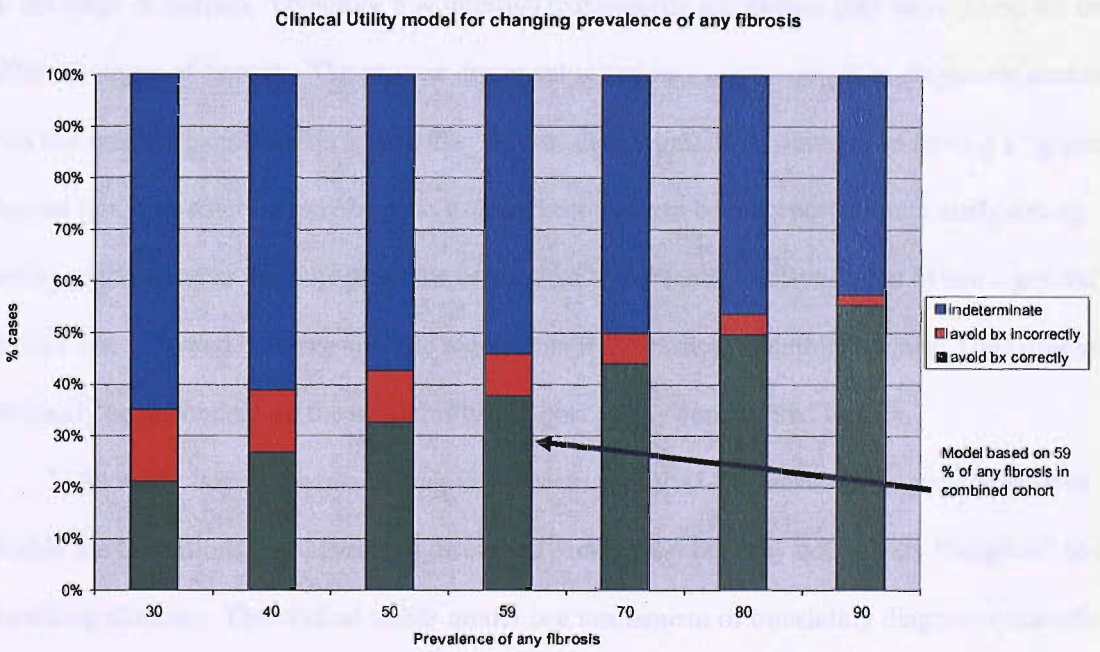


Figure 4-8: Clinical Utility Models showing effect of changing prevalence on diagnosis of any fibrosis



4.3 Discussion

The ELF panel had an excellent performance in distinguishing severe fibrosis in this validation data set with an AUC of 0.91. Simplification of the algorithm by removing age did not alter the diagnostic test performance. The addition of simple parameters to ELF increased diagnostic performance in distinguishing no fibrosis, moderate fibrosis and severe fibrosis. The most significant impact of the improved performance of the combined algorithm may be in distinguishing any fibrosis.

In the three derived simple panels, different parameters emerged but there are general similarities to the findings in the systematic review in chapter 3. Measures of glucose sensitivity, albumin and age feature in all three algorithms and these have face validity in measuring fibrosis (will discuss further in final chapter). The improvement by combining simple markers and ELF panel suggests that different aspects of fibrosis are being measured. It is interesting to speculate that the combined algorithms could be measuring factors associated with aetiology (eg insulin sensitivity), mechanisms of injury (eg matrix products in ELF) and functional consequences of fibrosis (eg albumin and insulin sensitivity). The balance of these constituents may vary depending on the stage of fibrosis. Therefore it is intuitive that specific algorithms may be required for the different stages of fibrosis. The counter argument is that any improvement in diagnostic accuracy with the specific panels needs to be offset against the pragmatic advantages of having a "general" fibrosis test. The solution may be a) to use markers that can be incorporated into analysers eg fasting glucose rather than the presence or absence of the metabolic syndrome b) use a general fibrosis test followed by more specific algorithms if the clinical situation dictates. The latter will obviously be dependent on the availability and cost of any commercial test.

Showing improvements in diagnostic accuracy by AUC measures has statistical merit (within the limitations that have been discussed previously) but may not be very "tangible" to the practising clinician. The clinical utility model is a mechanism of translating diagnostic statistics into clinical practice. The potential to avoid over 80 % of liver biopsies using the combined

algorithm for the detection of severe fibrosis has wide implications on patient management and health care resource.

There are some inherent limitations of the clinical utility model. Firstly it makes broad assumptions about certainty. There will be variability amongst clinicians about the level of diagnostic certainty they are satisfied with. Moreover, in clinical practice a multiple tools may be used for diagnosis eg clinical examination, blood tests, radiology, etc. The precise contribution of each of these parameters varies and is difficult to quantify. Therefore this modelling oversimplifies the diagnostic process. It is not clear what error rate would be acceptable to clinicians for the diagnosis of liver fibrosis, and conservative thresholds have been deliberately chosen; it could be argued that we could use larger error rates as the sampling error for liver biopsy may be as high as 30 % [159]. To ascertain these levels of certainty one could conduct a Delphi questionnaire on opinion leaders or perform a postal survey of potential clients of the test (eg hospital gastroenterologists or GPs); this is discussed further in the final chapter.

The effect of prevalence on test performance is important in trying to predict the application of non-invasive markers to clinical practice. Hitherto, the published literature on clinical practice has concentrated on higher prevalence population in secondary or tertiary care. There are a number of reasons for this including resource issues but the major determinant is likely to be the requirement of obtaining a liver biopsy (largely performed only in hospitalised settings) for these diagnostic studies. The use of serum markers in the community to stratify patients for referral, observation or reassurance has obvious advantages. The modelling suggests that the current markers could have a potential role in the community for the management of severe fibrosis but presently under perform for the detection of any fibrosis. There are a number of ways this could be improved, for example finding new markers to add to the algorithm and therefore strengthen diagnostic performance. Alternatively one could use diagnostics tests (eg liver function tests) in the community test before applying the serum marker test (essentially artificially increasing the prevalence of disease). The effect of spectrum bias cannot be modelled. To account for this a study in primary care would need to be performed but the issue of requiring a liver biopsy as a reference standard makes this a difficult ethical proposition.

The ELF panel can be used to distinguish different stages of fibrosis and this can be improved upon by the addition of simple markers. The clinical utility model exemplifies how the serum markers could be used in current clinical practice and in different prevalence setting of fibrosis. The continuing improvement of serum markers, particularly at the earlier stages of fibrosis, will increase their applicability. In the next part of the thesis I will explore how new technologies may aid the improvement of non-invasive markers.

Chapter 5 : Introduction to Metabonomics

Metabonomics is an emerging technology allied to the other "-omics" such as genomics (study of genes), transcriptomics (study of transcription products), proteomics (study of proteins) and lipomics (study of lipids). All of these technologies generate extremely large datasets which has a number of consequences. Firstly, the data generated is often hypothesis generating rather than hypothesis driven. This may demonstrate associations which may not have been considered to be influential. These technologies will not necessarily reveal causal relationships and therefore further studies are often required to illustrate mechanistic processes. Secondly, the large data sets pose an issue with analysis and due to the extremely large number of variables generated specialised statistical methods are required.

The study of genes and proteins is extremely valuable is understanding disease process and creating novel diagnostic and therapeutic strategies. However, it is not simply the presence or absence of a gene or protein that will influence events further "down stream" in a metabolic pathways. The activation of the gene, transcription into a protein product, transportation of that protein through the cell, secretion and post translational modification are all key steps. Metabonomics is the study of low molecular weight metabolites in a biological system and is influenced by genes but also external factors such as the environment, drugs and disease. It is therefore able to give a holistic snapshot of a biological system by analysing readily obtainable biofluids such as serum or urine. The metabolites have the potential to be amino-acids, proteins, carbohydrates or lipids. The term metabonomics is defined as "quantitative measurement of the time related multiparametric metabolic response of living systems to pathological stimuli".[160]

Physiological systems have wide biological variability, even in the absence of disease, eg changes related to diurnal variation, and this may be detected by metabonomics. Therefore when comparing disease to healthy subjects the difference in the signals of metabolites as a result of disease must be greater than those occurring as part of normal physiological variation. This concept

is often referred to as the signal to noise ratio and is important in designing and interpreting metabonomic studies.

5.1 Metabonomics in human disease

The majority of studies using metabonomics have concentrated on animal models and in particular the analysis of metabolites induced by drug toxicity. There have some studies on assessing metabonomics in human disease. Brindle et al. [161] used metabonomics to distinguish patients as having normal coronary arteries or triple vessel disease using angiography as the reference standard. The authors created a model on a training set and validated the model on a group of patients with a sensitivity 92 % of and specificity of 93 %. Furthermore, they were able to separate patients on the basis of one, two and three vessel disease. They were also able to hypothesise about biological plausibility as prominent peaks corresponded to specific lipid profiles.

A more recent study studied the role of metabonomics in the detection of epithelial ovarian cancer [162]. Using metabonomics they were able to separate 38 patients with epithelial ovarian cancer from 12 patients with benign ovarian disease. Furthermore, they were able to separate cancer patients with 100 % accuracy from premenopausal women and 97 % accuracy from post menopausal women.

Hitherto, there have been no published clinical studies using metabonomics to assess liver fibrosis. There has been a study that uses metabonomics to distinguishing liver cancer from hepatitis and cirrhosis [163] and a further study on finding biomarkers for acute deterioration in hepatitis B [164].

5.2 Principles of NMR in metabonomics

Nuclear magnetic resonance (NMR) spectroscopy was used in this project to detect metabolites. NMR utilises the fact that some atoms have a magnetic charge. This charge is influenced not only by the type of atom but also its location within the molecule and its chemical neighbours. The magnetic properties of certain atoms eg hydrogen and carbon make them suitable

for spectroscopy. When atoms are placed in a magnetic field, they will align in that field (see figures 5.1 and 5.2). Using an electric current at an angle to this magnetic field, molecules can be made to resonate at a precise frequency. This resonance and subsequent relaxation can be measured and transformed into spectral lines (see figures 5.3 and 5.4). In our clinical study NMR spectroscopy is based on hydrogen spectroscopy and allows us to measure the presence or absence of metabolites containing hydrogen. The molecules will have different spectral lines because of their size and magnetic properties and can therefore be distinguished.

Figure 5-1: NMR magnet

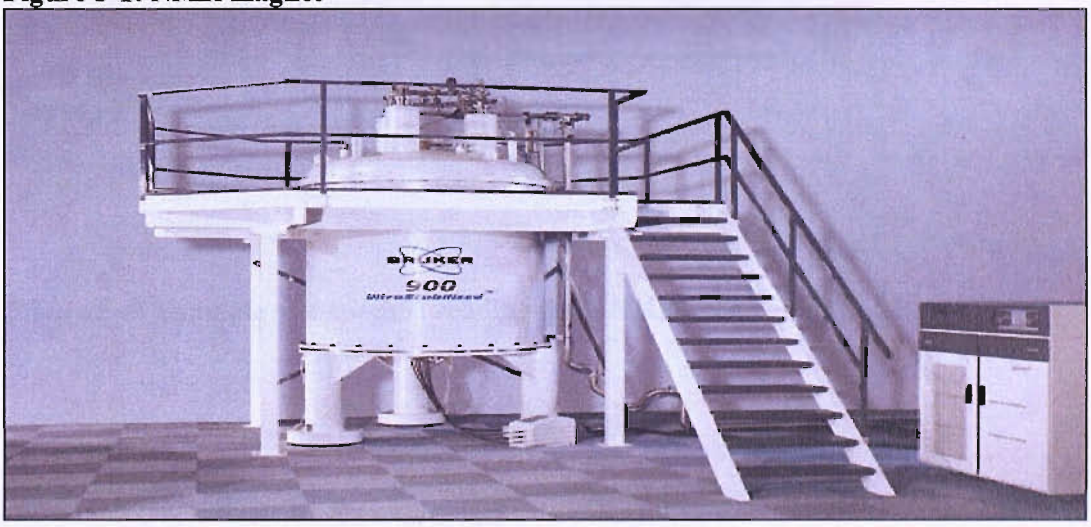


Figure 5-2: Diagram showing alignment of atoms in a magnetic field.

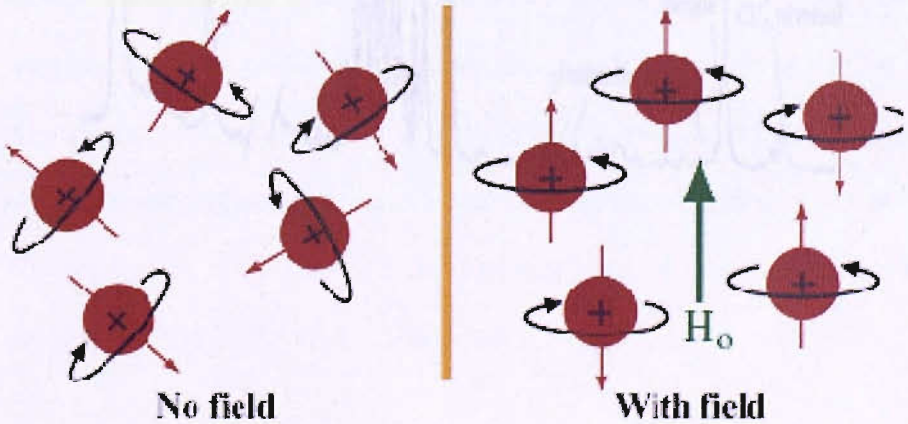


Figure 5-3: NMR sample acquisition

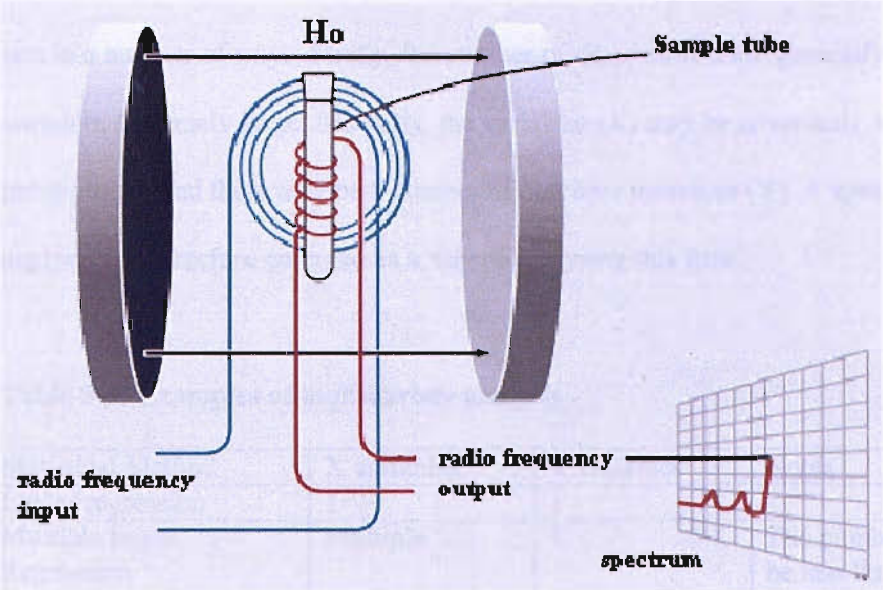
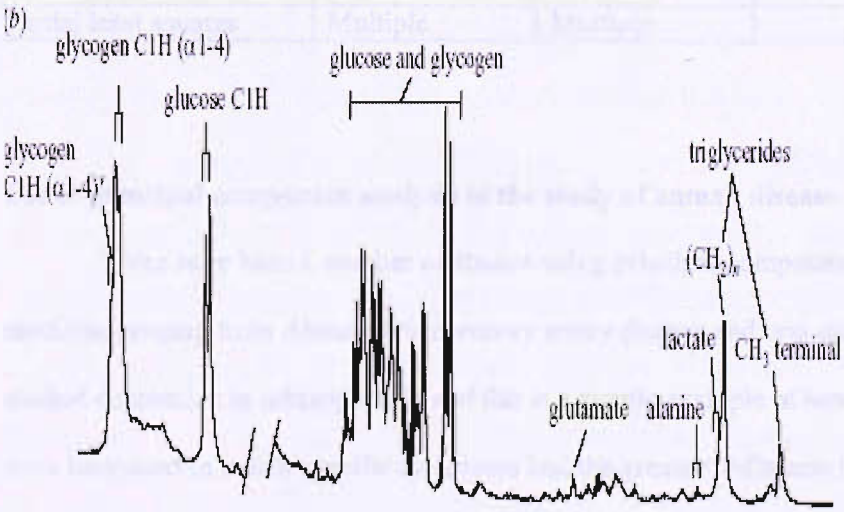


Figure 5-4: Example of a spectral reading from NMR spectroscopy.



Data analysis

The data generated by metabonomics differs from traditional data sets in a number of ways. Firstly, the number of observations are generally low but the number of variables extremely large. Secondly, the variables (X) may be covariants, which are not independent, and there may be a number of outcome measures (Y). A specific type of multivariate analysis has therefore emerged as a way of analysing this data.

Table 5-1: Examples of multivariate analysis

Statistical Method	X variables	Y variables	Notes
Linear regression	1	1	
Multiple linear Regression	Multiple	1	The number X variables should be less than number of observations. X variables should be independent
Principal components regression	Multiple	1	Number of variables can be greater than number of observations. Independence not necessary
Partial least squares	Multiple	Multiple	

Use of principal component analysis in the study of human disease

There have been a number of studies using principal components analysis (PCA) in medicine ranging from Alzheimers, coronary artery disease and oral disease. Maggini et al [165] studied depression in schizophrenia and this is a simple example of how PCA can be used. They were interested in which specific symptoms had the greatest influence in depression in the context of chronic schizophrenia and interviewed 342 patients using the Calgary Depression Scale (CDS). The relationship of variables with depression (diagnosed by a CDS score of greater than 6) was analysed by PCA and is shown in table 5.2.

Table 5-2: CDS item contribution to components (only factor loading greater than 0.5 shown)

CDS item	Component 1	Component 2	Component 3
C1 depression	0.80		
C2 hopelessness	0.77		
C6 morning depression	0.74		
C9 observed depression	0.73		
C3 self deprecation	0.69		
C8 suicide	0.63		
C4 guilty ideas of reference		0.87	
C5 pathological guilt		0.70	
C7 early morning wakening			0.955
Variance accounted for	43	13	12

This analysis illustrates a number of advantages of using PCA. 9 different variables can be condensed into three components. The individual components are unrelated as they are orthogonal to one another (explanation in next section). Each successive component explains reducing variability of data, 43 %, 13 % and 13 % respectively and in total the three components explain 68 % of the variation of the data. The key variables within each component can be elucidated. For example in component 1 depression, hopelessness, morning depression, observed depression, self deprecation and suicide all have high loading values and make a significant contribution to component 1. In contrast, early morning wakening accounts almost exclusively for factor 3. In biological studies there be many more variables and these may be unknown so the purpose of this analysis can be multifold. It can reduce the data set from over 100 variables into 2-4 principal components which can explain the data set, identify key variables which have greatest impact on one or more principal components (unsupervised analysis) and also create models using information from key variables to predict class identity (supervised analysis).

Principal component analysis in the analysis of metabonomic data

Principal components analysis is one of the central features of multivariate analysis. It is used to examine data in unsupervised manner. Each variable (or descriptor) is given a dimension in space (k space). Therefore an observation with 10 variables will have a unique plot in 10 dimensional space. Vectors can be generated in this multidimensional space showing patterns of correlation within the data set. These vectors, represented mathematically by eigen values, are known as principal components. Figure 5.5 shows hypothetical data points, in figure 5.6 the red line represents principal component 1; this represents the direction which best exemplifies the data set. A second principal component, shown by the green arrow in figure 5.7, is a vector at 90 degrees (orthogonal) to the first and is therefore independent. Further principal components can be created at continuing orthogonality (i.e. 180 degrees, 270 degrees, 360 degrees, 450 degrees, etc).

Figure 5-5: Hypothetical data set to illustrate principal components

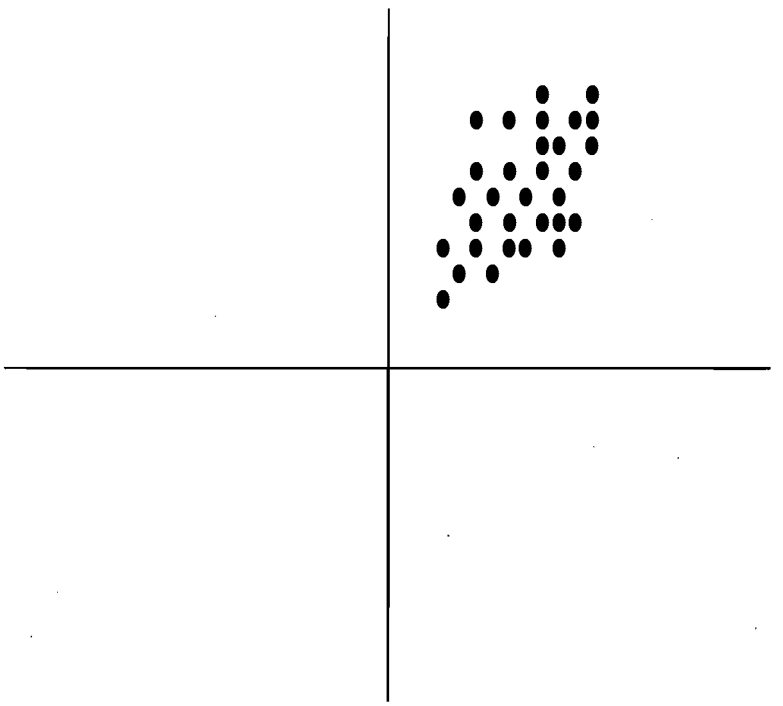


Figure 5-6: Hypothetical data showing first principal component

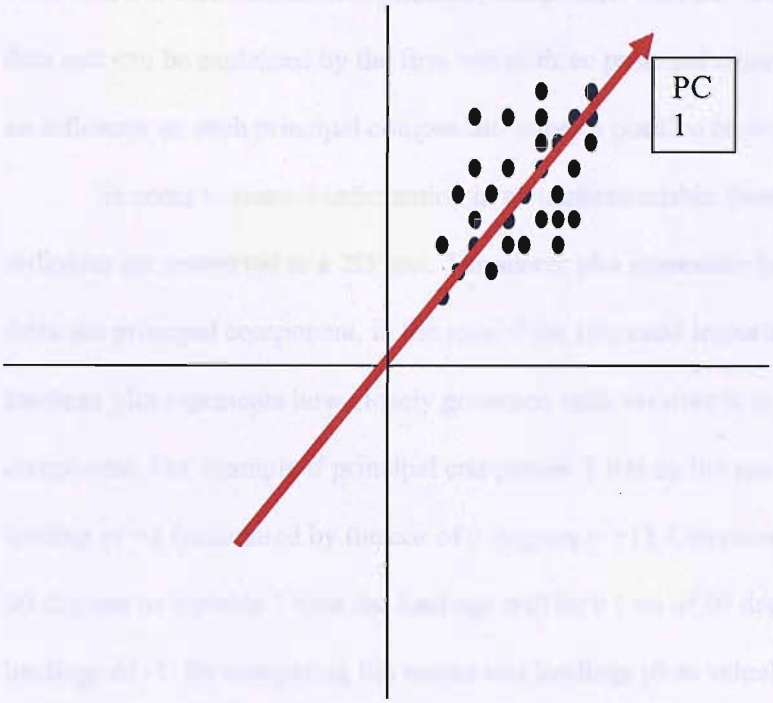
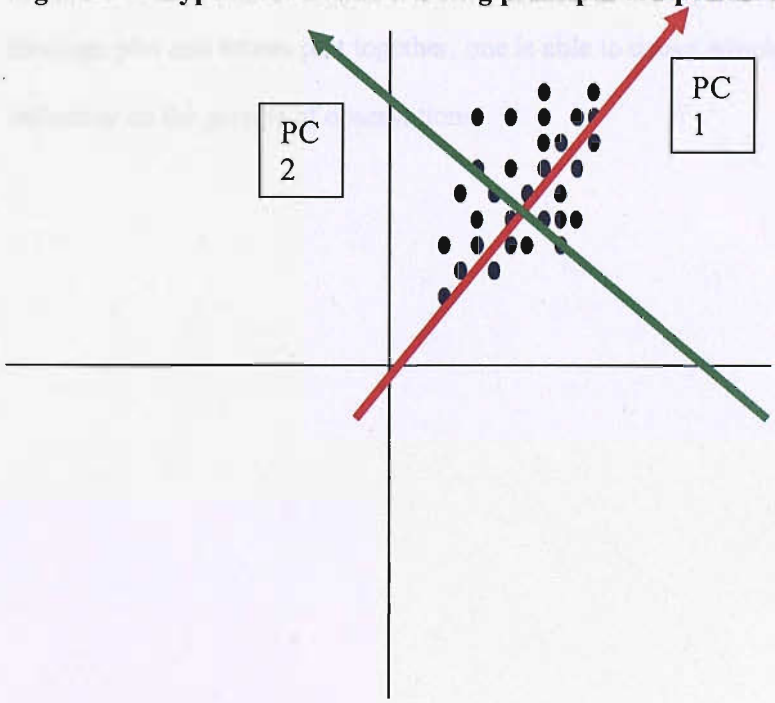


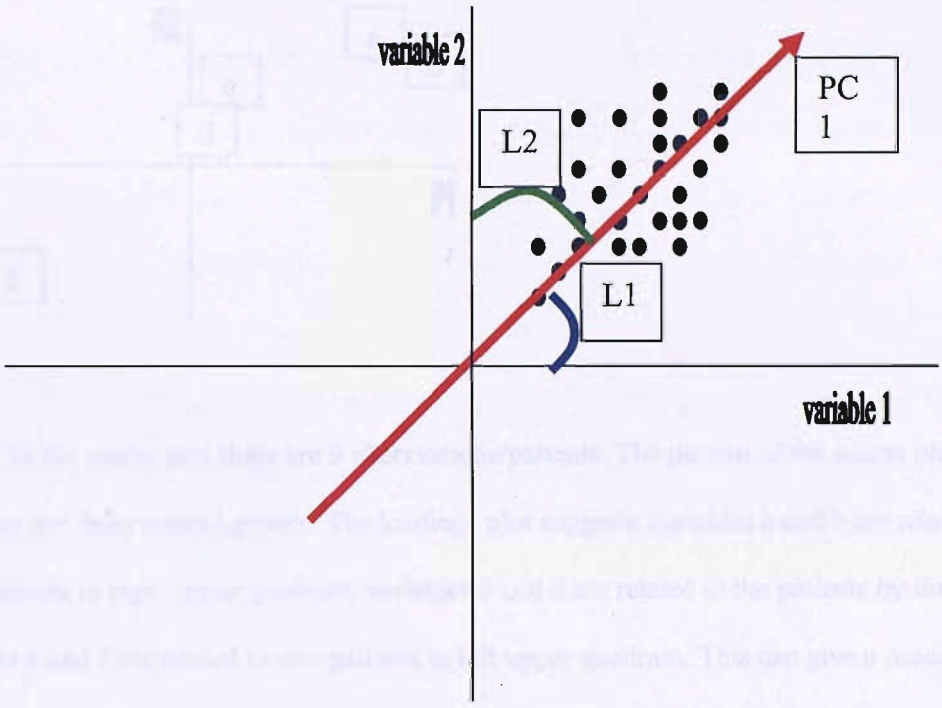
Figure 5-7: Hypothetical data showing principal component 1 and 2



The maximum number of principal components will be determined by the number of variables, but each consecutive principal component weakens in its ability to explain the data. Most data sets can be explained by the first two to three principal components. Each variable will have an influence on each principal component; either a positive or negative association.

In order to present information in an understandable form the theoretical mathematical co-ordinates are converted to a 2D plot. The scores plot represents how far each observation is away from the principal component, in the axis of the two most important variables (descriptors). The loadings plot represents how closely governed each variable is to its respective principal component. For example if principal component 1 lies on the same plane as variable 1 it is given a loading of +1 (calculated by the cos of 0 degrees = +1). Conversely if the principal component is 90 degrees to variable 1 then the loadings will be 0 (cos of 90 degrees = 0); 180 degrees will give a loadings of -1. By comparing the scores and loadings plots valuable information can be gathered. In figure 5.8, PC 1 is at a 45 degree angle to variable 1 represented by L1, thus has a loading weight of 0.5 (cos of 45 degrees =0.5) for variable 1. In relationship to variable 2, the angle is also 45 degrees represented by L2, thus the loading weight is also 0.5 for variable 2. By looking at a loadings plot and scores plot together, one is able to detect which variables are exerting the greatest influence on the groups of observations.

Figure 5-8: The relationship between variables and principal components



Below is a hypothetical example of a scores plot and loadings plot with 9 patients/ observations (labelled 1-9 in figure 5.9) and 6 variables (labelled a to f in figure 5.10).

Figure 5-9: Scores plot of 9 observation points

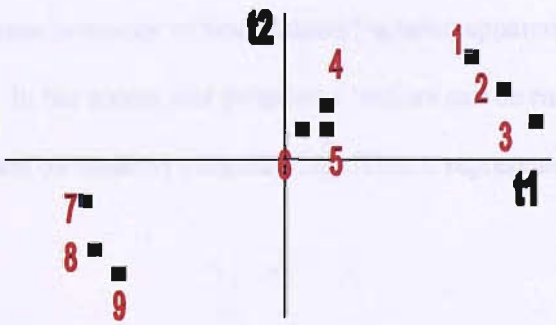
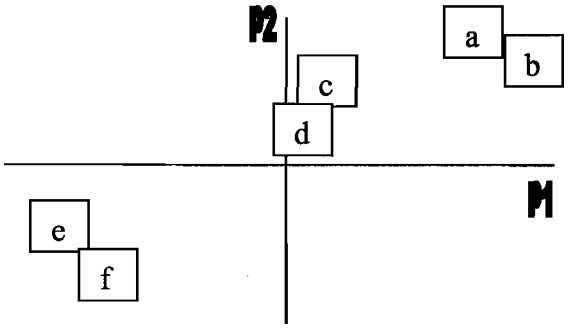


Figure 5-10: loadings plot of 9 observation points



In the scores plot there are 9 observations/patients. The pattern of the scores plot suggests that there are three natural groups. The loadings plot suggests variables a and b are related to the three patients in right upper quadrant, variables c and d are related to the patients by the origin and variables e and f are related to two patients in left upper quadrant. This can give a descriptive overview of the data. From the loadings plot, the variables a and b can be seen to have the greatest positive influence on the first two principal components and conversely variables e and f have a negative relationship with the first two principal components. Furthermore variables c and d lie close to the origin and do not help to distinguish the data set. This is a very simplistic example, and with such few variables there are better statistical methods for analysis. However, if one were to increase the number of variables to 100 or greater (not uncommon in metabonomics) the advantages/necessity of this analysis becomes apparent.

In the scores plot prominent outliers can be easily seen as they lie outside the Hotelling's T^2 (based on students t-distribution). This is represented by an elliptical circle within the scores plot.

R and Q values

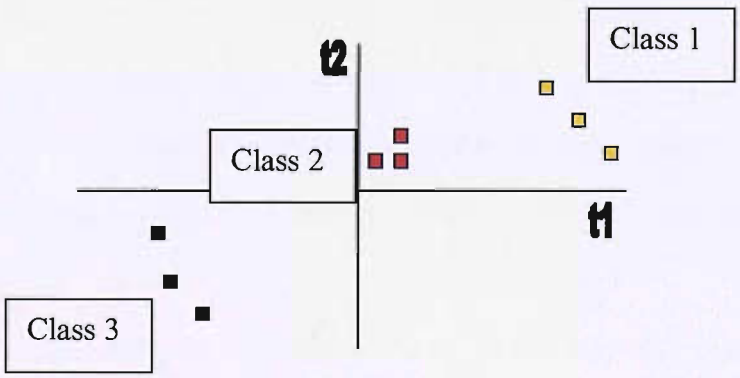
In most data sets the first two principal components (PC) explain the majority of the variation within the data set. However by increasing the number of PC more variation can be explained. The corollary to this is that there is a danger of "over fitting" the data and patterns become more specific and less generalisable. To summarise this the terms R^2 and Q^2 are used. R^2

refers to how well the model explains variation and is analogous to R^2 in regression analysis. It is based on the formula: R^2= 1- Σ (observed – fitted)^2 / total sum of squares. With increasing number of PC the R^2 value increases. The Q^2 value is derived from removing observations (eg 1/7 th of the total data set) and calculating a quotient based on predicted values from the model and the real "observed" value; formula Q^2= 1- Σ (observed – predicted)^2 / total sum of squares. Initially the Q^2 value will increase with PC but at a critical number of PC will decrease once the data starts to become over fitted. Excellent models have values of Q^2 of 0.9 but in metabonomics they are usually around 0.5; a value of 0.5 for R^2 suggests that 50 % of the variation can be explained by the model (ideal value is 1). Therefore the decision on how many PC are required in the final model is based on the balance of R^2 versus Q^2.

Supervised methods

Using PCA alone is useful in data where class information is unknown. If classes are known a priori then more detailed analysis can be undertaken. There are a number of methods but the main methods are soft independent modelling of class analogy (SIMCA) or partial least squares discriminant analysis (PLS DA). This creates models to discriminate classes and also explains why there are differences between classes. Using the hypothetical study in the unsupervised example, if we knew the classes of the 9 patients a priori, the scores plot would look like figure 5.11 below.

Figure 5-11: Scores plot of 9 observations which belong to three classes



PLS DA will not only separate classes in the scores plot, but the variables responsible for the separation can be obtained eg what variables separate yellow from red and red from black by

looking at a PLS-DA coefficients plot. PLS-DA utilises principal components that not only explain the variation in X but also account for the variation in Y. To check correct classification, new data is inserted and tested (external validation) or if new data is unavailable a proportion of the data set is omitted (internal validation). A dummy variable is assigned to each class, and it will be based on variables (placed in regression equations) that produce maximal separation. Therefore the value of the dummy variable may be 0 for class A and 1 for class B. An unknown observation can be put in the model; depending on how close the predicted value is to 0 or 1 it will be assigned to class A, class B or be unclassified.

Chapter 6 : Exploring metabonomics in liver fibrosis

As outlined in the previous chapters, serum markers have many potential advantages. They can be measured using simple blood tests, are relatively inexpensive compared to biopsy and have the potential for serial measurement. The identification of novel biomarkers, particularly at the earlier stages of disease, is a potential way of improving the non-invasive diagnosis of liver fibrosis.

There are a number of reasons why metabonomics could be a valuable tool in liver fibrosis. Firstly, it has the potential to be performed on serum or urine samples. Secondly, the serum markers have concentrated on using known mediators or products of fibrosis in the diagnostic test. Using a hypothesis generating approach novel biomarkers may be found which aid diagnostic accuracy.

Metabolites may only be detectable after a certain stage of fibrosis is reached (threshold of detection) or alter as a continuous variable. The challenge will be to find stable metabolites that have minimal "physiological" variation. They will need to be produced constantly or have a long half life to allow diagnostic utilisation and demonstrate consistent changes in pattern detection with injury and fibrosis. Ideally this pattern will allow the differentiation of not only significant fibrosis or cirrhosis from normal but also identify more subtle forms of fibrotic injury.

Hepatitis C was chosen as the disease aetiology as this condition has been most extensively studied using serum markers (as outlined in the previous chapters), a robust diagnostic test is available for the disease aetiology (viral levels can be measured directly in blood) and liver biopsies (representing the current reference standard for diagnosis) were readily available in this aetiology of liver disease at the start of the study (national guidelines changed during the course of the thesis).

To determine if metabolites found in the clinical study were specific to the aetiology of injury a parallel study was performed in an animal model of liver fibrosis following injury with carbon tetrachloride (CCl^4).

Aims

- To discover biomarkers in serum and urine in a human study of liver fibrosis secondary to hepatitis C, using metabonomics and NMR spectroscopy.
- To discover biomarkers in hepatic tissue in an animal model of liver fibrosis secondary to CCl^4

6.1 Methods

Clinical study

This was a prospective, cross sectional study including patients with CHC and healthy controls (HC). Subjects were recruited from two centres, Southampton General Hospital and the Royal Bournemouth Hospital (LREC no. 04/Q1701/58). Inclusion criteria are shown in table 6.1 and include serological evidence of CHC and recent liver biopsy. The exclusion criteria are shown in table 6.2 and include confounding concurrent chronic disease, treatment with interferon and ribavarin and co-infection with hepatitis B (HBV) or human immunodeficiency virus (HIV).

Table 6-1: Inclusion criteria for study

Inclusion criteria:
• Male and female subjects aged 18-65 with CHC
• Evidence of liver fibrosis, confirmed by routine liver biopsy taken between 2 weeks and four months (healthy controls did not undergo liver biopsy).
• Demonstrable HCV viraemia at least six months prior to entry

Table 6-2: Exclusion criteria for study

Exclusion criteria:

- Presence of autoimmune disease, osteoarthritis, rheumatoid arthritis, inflammatory bowel disease, chronic obstructive pulmonary disease, hypertension, renal fibrosis, scleroderma, diabetes mellitus, heart failure, recent myocardial infarction (last 12 months), unstable angina and carcinoma
- Renal impairment indicated by Creatinine >130 mmmol/l
- Coinfection with HIV or HBV
- Previous treatment for HCV with antiviral agents
- Evidence of secondary aetiology of liver disease on biopsy
- On medication which cannot be stopped one week before study

Sample collection

Eligible subjects were given information about the study (see appendix 7). Volunteers (who had at least 24 hours to consider their decision) were invited to attend an appointment at the Wellcome Clinical Trust Research Facility (WTCRF) at Southampton General Hospital. In the information sheet, patients were requested to avoid herbal remedies, dietary supplements and non-essential medication in the week prior to the study visit. Additionally, they were asked to avoid fish, seafood, spicy foods or alcohol on the day before and the day of the study. The reasons for this are because previous studies have shown that these items can influence metabonomic signals. All samples were collected between 9 am and midday.

After consent had been obtained, patients underwent a brief history and clinical examination. 50 mls of blood was venesected for baseline blood tests (urea and electrolytes, full blood count, clotting and liver function tests), viral load and genotype, serum markers of liver fibrosis (ELF test) and metabonomic assessment. Blood for metabonomic assessment was collected in EDTA and Lithium Heparin tubes and chilled immediately on ice. Centrifugation was performed within 30 minutes and samples were divided into 10 aliquots and stored at -80 degress. 50 mls of

urine was collected for metabonomic assessment; following centrifugation samples were stored at -80 degrees. The samples were shipped for analysis at the NMR facility, Pfizer, Sandwich, Kent and acquisition undertaken in conjunction with an NMR physicist (OB).

Animal study

Liver injury was induced in Sprague Dawley rats as described previously[166]. Briefly cohorts of 4 animals were injected intra-peritoneally with 0.2 ml/100 g sterile CCL_4 dissolved in a 1:1 ratio with olive oil twice weekly for 4 and 8 weeks respectively to generate a reversible fibrosis and reversible cirrhosis respectively. In addition a cohort was treated for 12 weeks to establish advanced micronodular cirrhosis which undergoes only partial resolution over 1 year of follow –up (irreversible cirrhosis). For each model, animals were sacrificed and livers harvested 72 hours after the final injection of CCL_4. Four, untreated rat livers were also harvested for use as controls. Liver tissue provided by TK. Harvested livers were snap frozen in liquid nitrogen for metabonomic analysis.

Liver biopsy

All clinical liver biopsies were assessed by a single pathologist using the Metavir scoring system. Biopsies were included if they were greater than 15 mm in length and contained more than 6 portal tracts. Histology is a surrogate for the future prognosis of liver disease and previous studies have suggested that fibrosis can be divided into three categories [84]. In this study a three stage classification was chosen; no fibrosis (class 1) versus mild/moderate fibrosis (class 2) versus severe fibrosis (class 3) corresponding to the Metavir stages HC/F0 versus F1/F2 versus F3/F4.

Rat liver tissue was fixed in formaldehyde, processed, embedded in paraffin wax and sectioned. Sections of each liver were stained with standard haematoxylin and eosin (H and E) and Sirius red to allow morphological examination. A modified scoring system based on the presence and distribution of large fibrotic bands was used to assess fibrosis[167] . Three stages of classification were chosen, class 1- normal liver (control animals), class 2-early fibrosis (week 4 and week 8 models) and Class 3- cirrhosis (week 12). This division is based on the fact the week 12 model

shows irreversible injury after spontaneous recovery whilst the 4 and 8 week model show complete resolution of fibrosis after spontaneous recovery [166].

^{1}H-NMR spectroscopy

Clinical study

Sample preparation

Li-heparinised plasma was prepared by mixing 170 ml of plasma with 340 ml of 0.9% saline (containing 10% D_2O v/v). Urine was prepared by mixing 340 ml of urine with 170 ml of 0.2M phosphate buffer (Na_2HPO_4 and NaH_2PO_4) in water (incl. 20% D_2O v/v), containing 1 mM 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (Sigma-Aldrich - Gillingham, UK) as a reference standard and 3 mM NaN_3. The resulting pH was 7.4-7.5.

NMR spectroscopy

Samples were analysed on a VARIAN Inova (Varian Inc., Scientific Instruments - Yarnton, UK) 500 MHz spectrometer using a 5 mm Varian 500 ID PFG probe head operating at 300K. For plasma, a CPMGpr sequence was used to remove short T_2 components with 128 transients, 32,000 data points and a spectral width of 12,000 Hz. For urine samples a noesypr1d pulse sequence with 2s presaturation delay and 100ms mixing time was used (64 transients, 32,000 data points, spectral width of 10,000 Hz). Prior to Fourier transformation a line broadening factor of 1 Hz was applied to both plasma and urine spectra.

NMR data processing

The NMR spectra in plasma were phased, baseline corrected and referenced to the glucose H1 proton at 5.23 ppm using Pfizer software.The spectra were divided into areas of equal width (0.04 ppm) from 10.0 to 0.2 ppm. This so-called data 'bucketing' simplifies statistical analysis and reduces the impact of small variations along the chemical shift axis. The integrals of the individual buckets per spectrum were area-normalised to the total area of each corresponding spectrum, excluding the area of the water from 5.2-4.2 ppm (CPMGpr). In urine, the NMR spectra were phased, baseline corrected and referenced to the 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP)

singlet at 0 ppm using in-house software. The spectra were divided into areas of equal width (0.04 ppm) from 10.0 to 0.2 ppm; excluding the area of the water from 6.2-4.42 ppm.

Animal study

Sample preparation

Liver tissue was analysed by three techniques a) magic angle spinning NMR (MAS-NMR) spectroscopy of the intact sample b) liquid phase NMR on water extracts and c) liquid phase NMR on lipid extracts. These data were acquired by Pfizer (details of methodology are given in appendix 8) and raw data then provided for analysis in this thesis.

Statistical analysis

Chemometrics analysis was performed using either Pfizer software written in MATLAB or SIMCA-P software (version P+ 11.0 and P 10.0, Umetrics AB, Umea, Sweden). All data was mean centred and pareto scaled. Spectral data from urine and plasma was combined into one database for the clinical study. Spectral data was analysed separately for the different methods in the animal model. Multivariate analysis was performed on NMR spectral data using unsupervised analysis (principal components analysis) and supervised analysis (partial least squares discriminant analysis (PLS-DA)). In the animal and clinical study analysis was performed on class 3 vs class 1, class 1 vs class 2 and class 2 vs class 3 using the first three principal components.

Validation of models

To test each model, internal cross validation was performed. In each model two observations were omitted by random selection and a new model created with the residual observations. The resulting model was the used to predict which class the omitted observations should belong to. This was repeated until all the observations had been omitted at least once. The prediction could have three outcomes, classification in the correct class, incorrect classification or assignment not possible.

Identification of key metabolites

Key spectral areas for each model were identified by ranking the top twenty variables for each model by its Coefficent CS value, analogous to the regression coefficient used in linear regression and is determined by the contribution of the variable to the principle component (loading). The spectral areas were then analysed individually to determine the major peaks within them. Metabolites were identified by comparing signature signals to known metabolites listed in reference NMR databases. For all identified metabolites (see tables 3 and 4), the area of signal intensity was calculated using in-house software. Univariate analysis was performed on selected signal intensities of identified compounds and tested using the Jonckheere-Terpstra test (analogous to Kruskal-Wallis but more appropriate for comparing ordinal, non-parametric groupings).

Translating the metabolic profile into a diagnostic algorithm in the clinical study

To produce a diagnostic algorithm for the clinical study, the key mediators identified in serum and urine were examined. The final algorithm contained only significant variables ($p<0.1$) remaining after forward logistic regression, based on the separation of moderate fibrosis. ROC statistics were produced for distinguishing no fibrosis, moderate fibrosis and severe fibrosis for this novel panel and compared to the established Enhanced liver Fibrosis (ELF) panel.

6.2 Results

Clinical study

Blood and urine were collected on 50 subjects. NMR spectroscopy was not possible on 4 urine samples because of interference of the water signal with adjacent spectral regions in these samples (very dilute samples) and two subjects were excluded from the healthy controls because of abnormalities in baseline biochemistry. Complete serum data was available for 50 subjects and complete urine data for 46 subjects. Baseline characteristic of the patients are shown in table 3. Variables that were statistically different between the classes ($p<0.05$) include age, AST, ALT, platelets and albumin. There was no statistical difference in the distribution of viral genotype between the three classes.

Table 6-3: Subject characteristics subdivided into no fibrosis, mild fibrosis and severe fibrosis

	Class 1 No fibrosis	Class 2 Moderate fibrosis	Class 3 Severe fibrosis
Number included	16	21	13
Male (%)	75 %	76%	77 %
Age	31.5 (30.0-44.3)	38.0 (31.5-42.0)	51.0 (40.0-57.5)
BMI	24.1 (19.7 -27.5)	24.5 (22.0 -27.4)	22.5 (20.6 - 24.6)
AST	20.0 (15.0 - 30.0)	42.5 (35.0 - 68.0)	57.0 (35.0 - 126.5)
ALT	34.5 (20.3- 42.5)	84.5 (59.3 – 260.5)	107.0 (76.0 -133.5)
Platelets	235.0 (206.8 - 291.3)	215.0(156.3-257.3)	159.5(104.8-216.8)
Albumin	42.0 (42.0-45.8)	42.0 (39.5- 43.8)	39.0 (36.5 to 43.5)

Footnote- Data presented as median values (inter quartile range in brackets) unless stated.

Results of spectral analysis in clinical study

The data were analysed by comparing each class against the other resulting in three analyses: class 3 vs class 1, class 1 vs class 2 and class 2 vs class 3. All the analyses presented use the first three principal components.

Serum

Class 3 vs class 1

Unsupervised analysis shows no clear distinction of the groups, although there does appear to be a trend of class 3 members lying to the right and class 1 members lying to the left of the scores plot (see fig 6.1). The loadings plot (fig 6.2) suggest the variables at the extreme right hand side (eg b3_42 and b3_46) have greatest influence on class 3 and the variables lying to the extreme left (eg b1_26 and b1_30 have greatest influence in class 1 variables. In the supervised analysis, using partial least squares discriminant analysis (PLS-DA) a model has been built with prior

knowledge of class assignment. This results in better discrimination of the classes as seen in figure 6.3. Members of class 3 and class 1 appear to separate on a line dividing the upper right hand quadrant and bottom left hand quadrant. The PLS DA model explains 85 % of the variation due to differences in fibrosis (R^2Y =0.85).

Class 2 vs Class 1

The supervised PLS DA model (fig 6.4) shows some separation of class 2 and class 1; this separation does not appear to be as good as class 3 vs class 1. The PLS DA model explains 71 % of the variation due to difference in fibrosis (R^2Y =0.71).

Class 2 vs Class 3

The supervised PLS DA model (fig 6.5) shows some separation of the two classes; the majority of class 3 members are clustered on the right. The separation of class 3 vs class 2 is the weakest, the model explains 55 % of the variation due to difference in fibrosis (R^2Y =0.55).

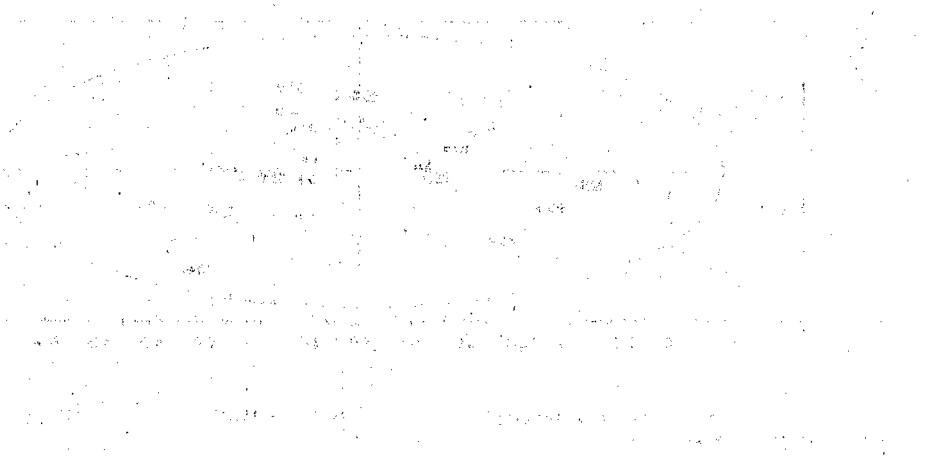


Figure 6-1: PCA - class 3 vs class 1 scores- serum

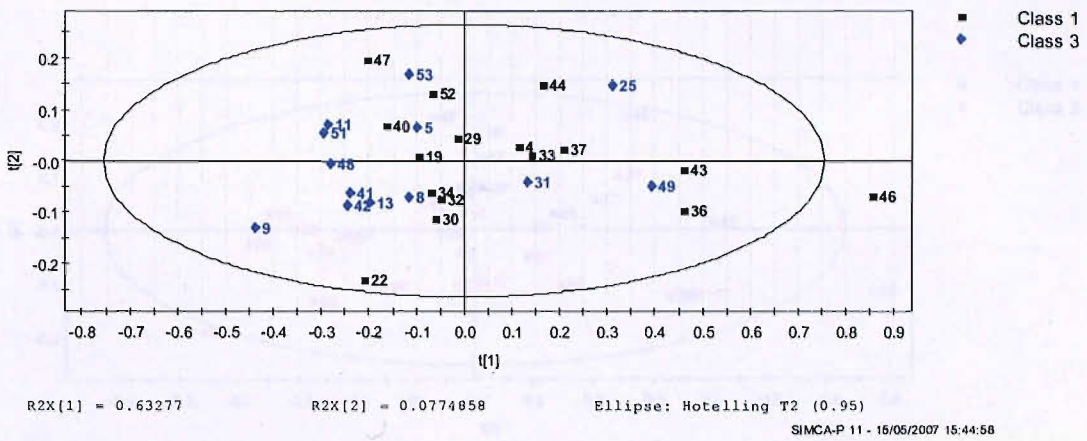


Figure 6-2: PCA - class 3 vs class 1 loadings- serum

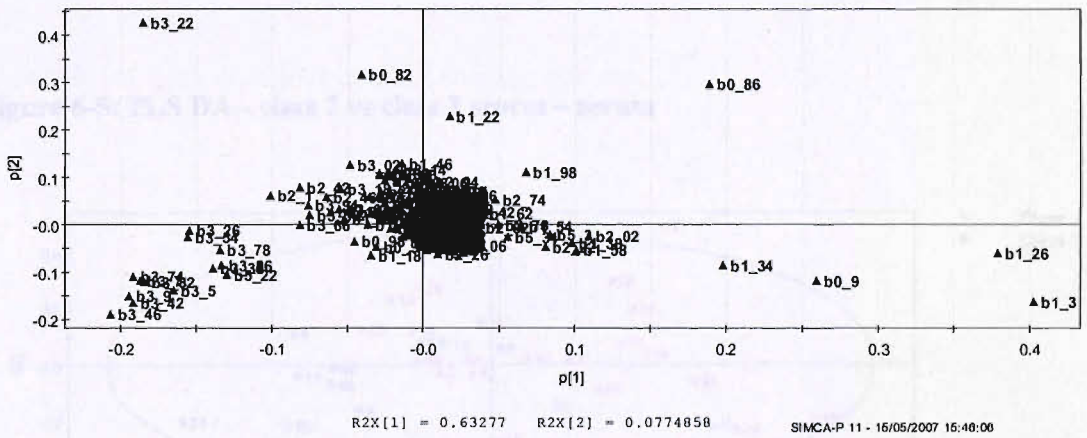


Figure 6-3: PLS DA - class 3 vs class 1 scores – serum

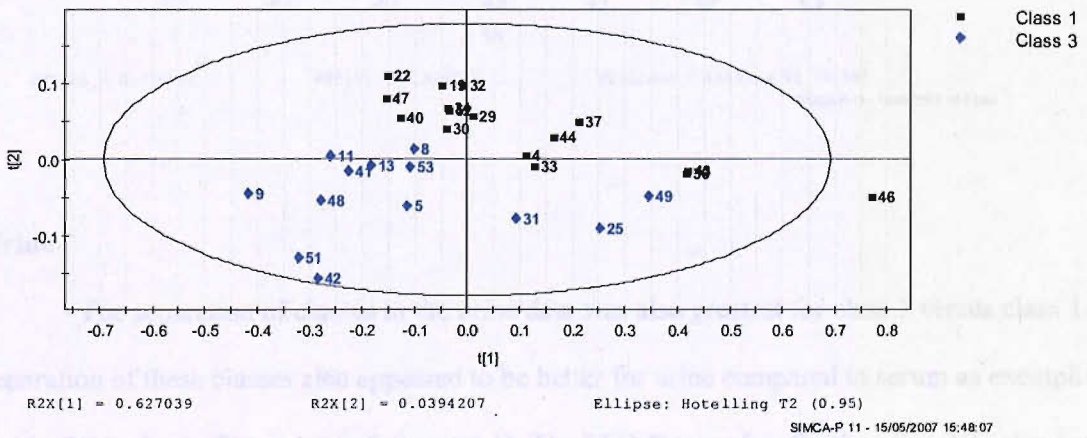


Figure 6-4: PLS DA - class 1 vs class 2 scores- serum

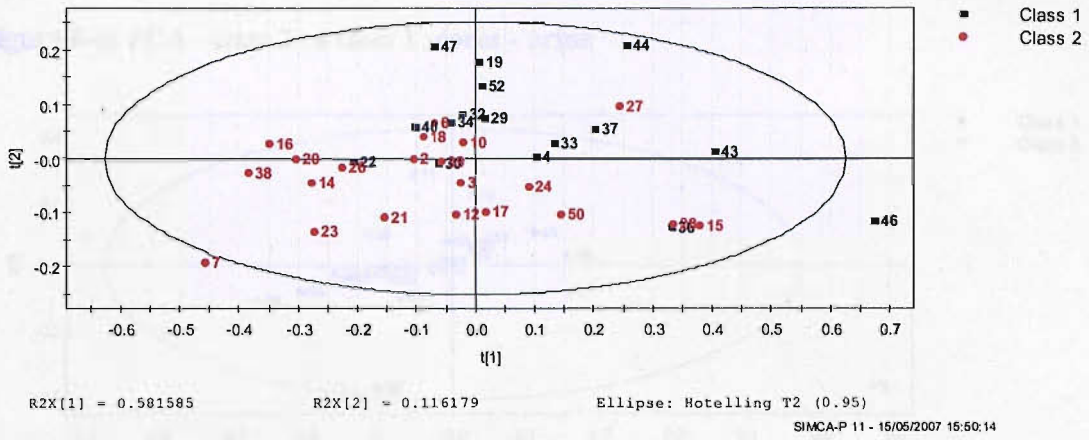
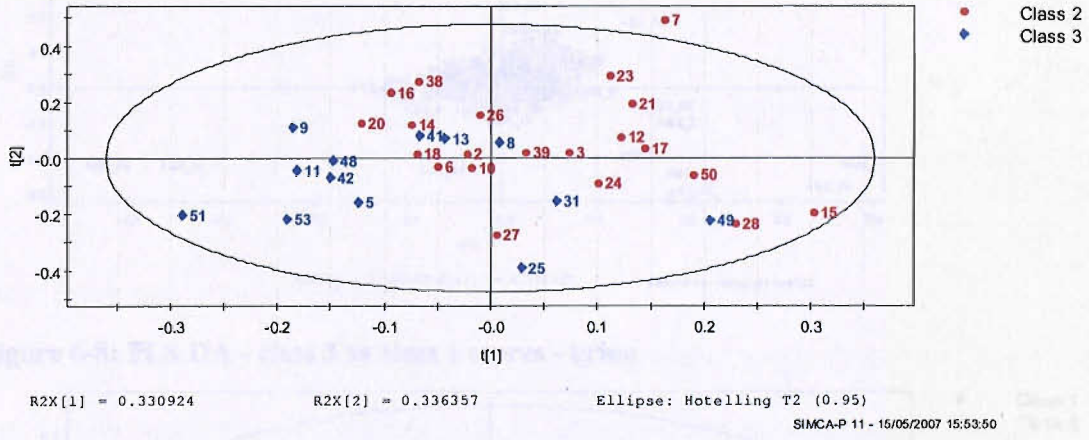


Figure 6-5: PLS DA - class 2 vs class 3 scores – serum



Urine

The separation of classes in the urine data was also greatest for class 3 versus class 1. The separation of these classes also appeared to be better for urine compared to serum as exemplified by the PCA plot in figure 6.6 (c.f. figure 6.1). The PLD DA models for the urine data also had higher R^2Y values for the separation of all three classes as follows: class 3 versus class 1 (R^2Y

=0.925), class 1 versus class 2 ($R^2Y = 0.532$) and class 2 versus 3 ($R^2Y = 0.629$); see figures 6.8 to 6.20.

Figure 6-6: PCA - class 3 vs class 1 scores - urine

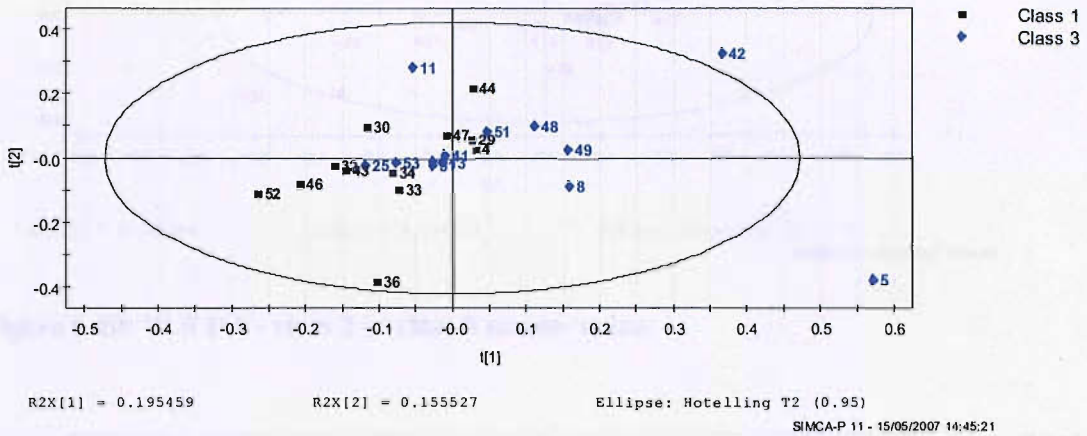


Figure 6-7: PCA - class 3 vs class 1 loadings - urine

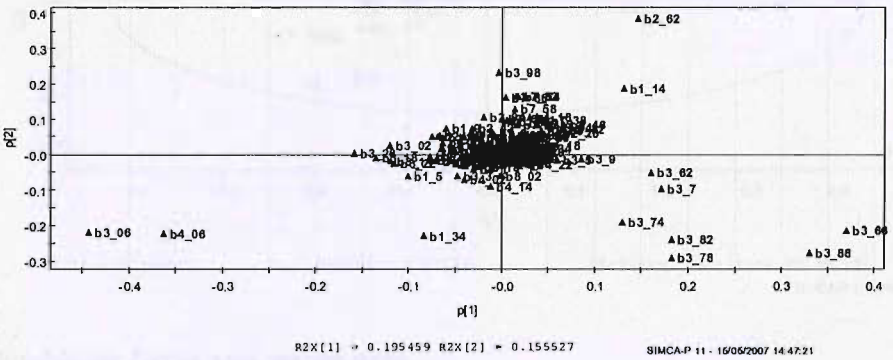


Figure 6-8: PLS DA - class 3 vs class 1 scores - urine

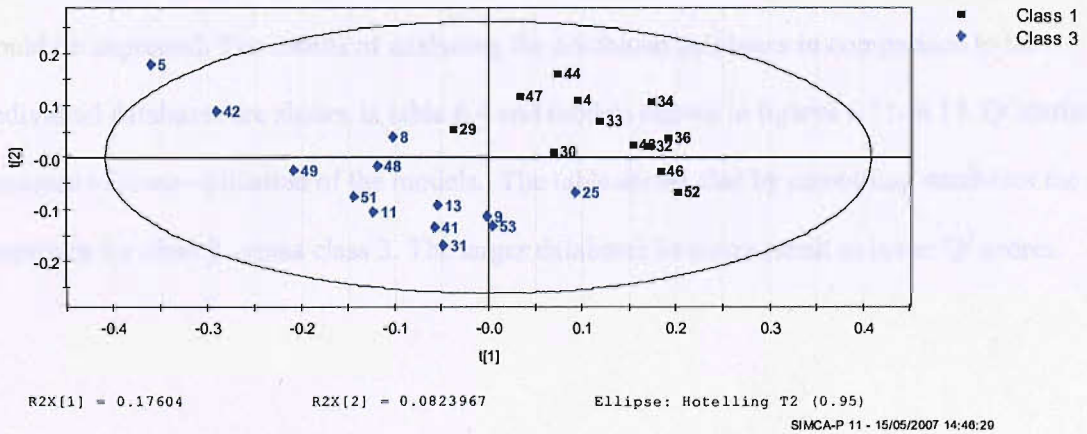


Figure 6-9: PLS DA - class 1 vs class 2 scores- urine

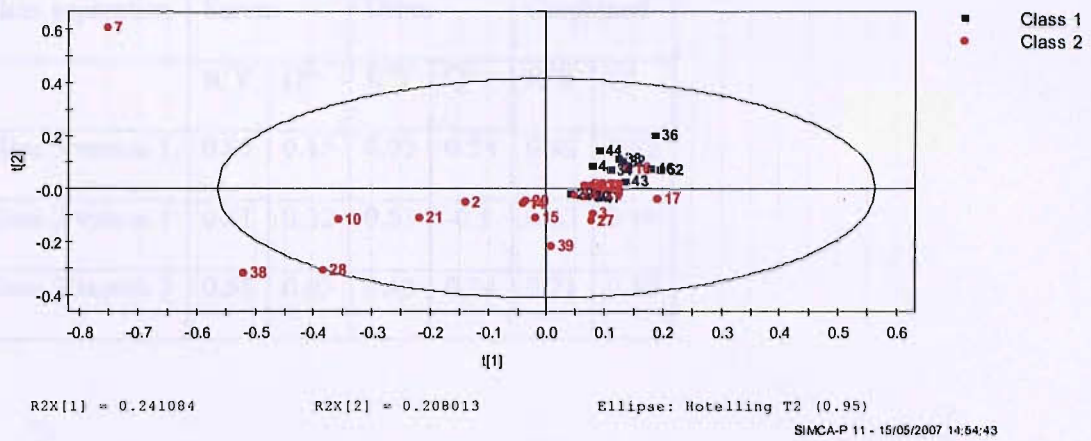
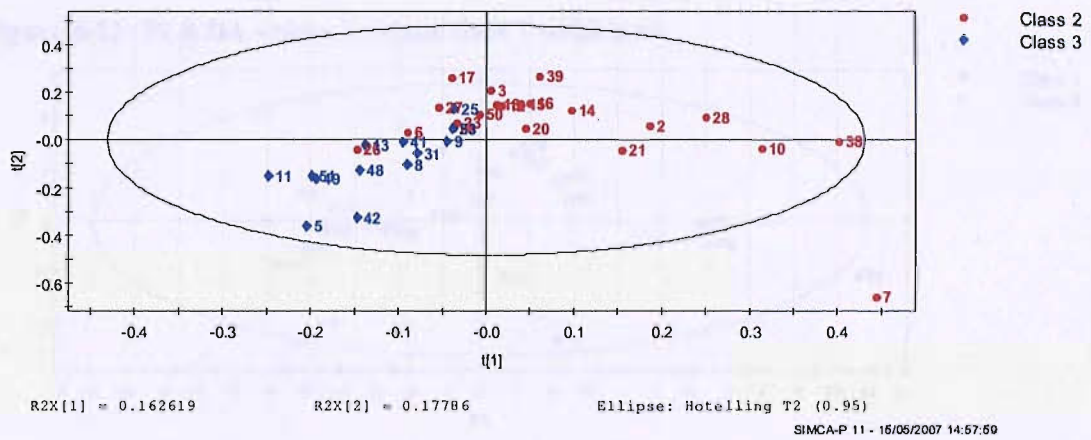


Figure 6-10: PLS DA - class 2 vs class 3 scores- urine



Combining Urine and serum data

The urine and serum NMR data were combined to see if separation of the stages of fibrosis could be improved. The results of analysing the combined databases in comparison to the individual databases are shown in table 6.4 and models shown in figures 6.11- 6.13. Q^2 statistic is a measure of cross-validation of the models. The table shows that by combining databases the model improves for class 2 versus class 3. The larger databases however result in lower Q^2 scores.

Table 6-4: Comparison of models produced by serum, urine and combined databases.

Class separation	Serum		Urine		Combined	
	R^2Y	Q^2	R^2Y	Q^2	R^2Y	Q^2
Class 3 versus 1	0.85	0.15	0.93	0.54	0.92	0.52
Class 2 versus 1	0.71	0.32	0.53	-0.1	0.63	0.10
Class 2 versus 3	0.55	0.03	0.63	0.34	0.71	0.10

Combined urine and serum database models

Figure 6-11: PLS DA - class 3 versus class 1 combined

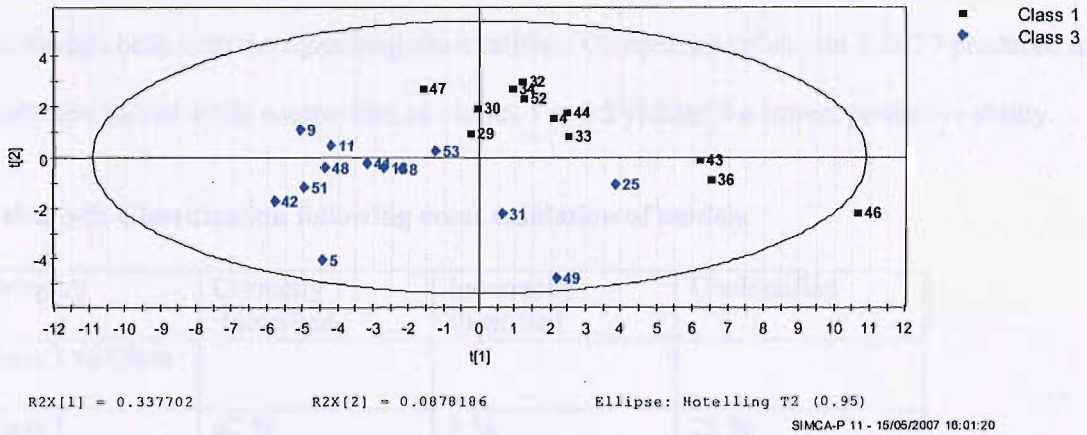


Figure 6-12: PLS DA - class 2 versus class 3 combined

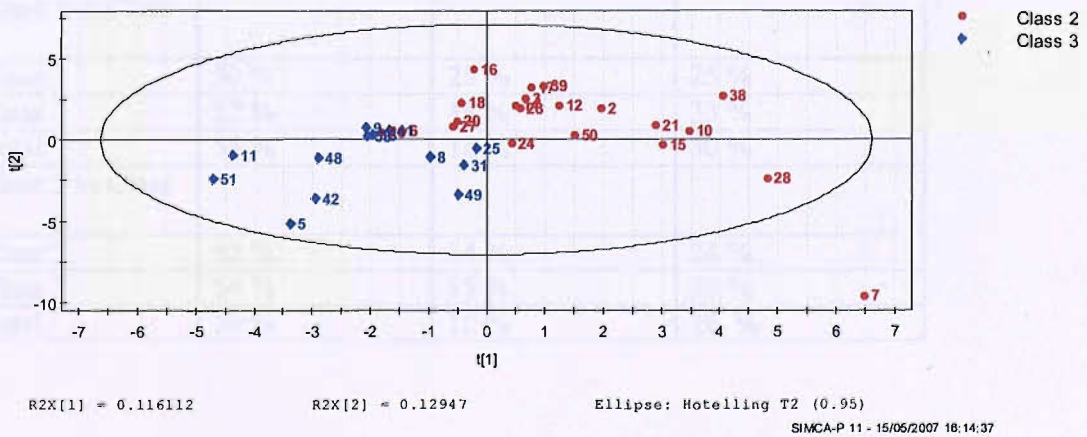
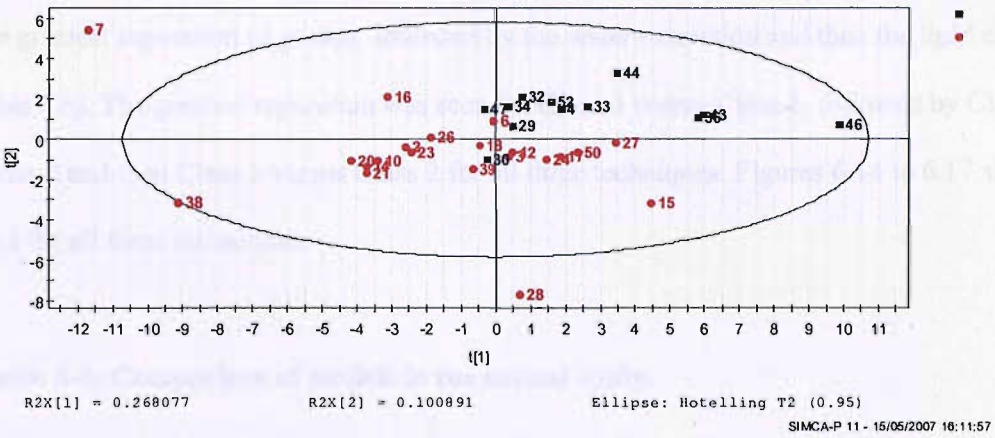


Figure 6-13: PLS DA - class 1 versus class 2 combined



Validation of combined serum and urine models in clinical study

The internal validation suggested that the majority of observations could be predicted by the models built with the remaining observations. Comparison of classes 1 and 3 produced the best predictive model while comparison of classes 1 and 2 yielded the lowest predictive ability.

Table 6-5: Classification following cross validation of models

Category	Correctly Identified	Incorrectly Identified	Unclassified
Class 1 vs Class 3			
Class 1	67 %	8 %	25 %
Class 3	62 %	15 %	23 %
Total	68 %	12 %	20 %
Class 1 vs Class 2			
Class 1	50 %	25 %	25 %
Class 2	52 %	14 %	33 %
Total	53 %	18 %	30 %
Class 2 vs Class 3			
Class 2	62 %	14 %	24 %
Class 3	54 %	15 %	30 %
Total	59 %	15 %	26 %

Results of animal study

Histology of the rat liver tissue (n=16) confirmed that longer duration of exposure to CCL_4 was associated with more severe liver injury. The magic angle spinning (MAS) analysis showed the greatest separation of groups, followed by the water extraction and then the lipid extraction (see table 6.6). The greatest separation was seen for Class 3 versus Class 1, followed by Class 1 versus Class 2 and then Class 3 versus Class 2 for all three techniques. Figures 6.14 to 6.17 show scores plot for all three techniques.

Table 6-6: Comparison of models in the animal study.

Class separation	Magic angle spinning		Water extraction		Lipid extraction	
	R^2Y	Q^2	R^2Y	Q^2	R^2Y	Q^2
Class 3 versus 1	0.97	0.88	0.97	0.91	0.92	0.49
Class 2 versus 1	0.96	0.67	0.92	0.68	0.77	0.22
Class 2 versus 3	0.84	0.42	0.8	0.16	0.75	0.18

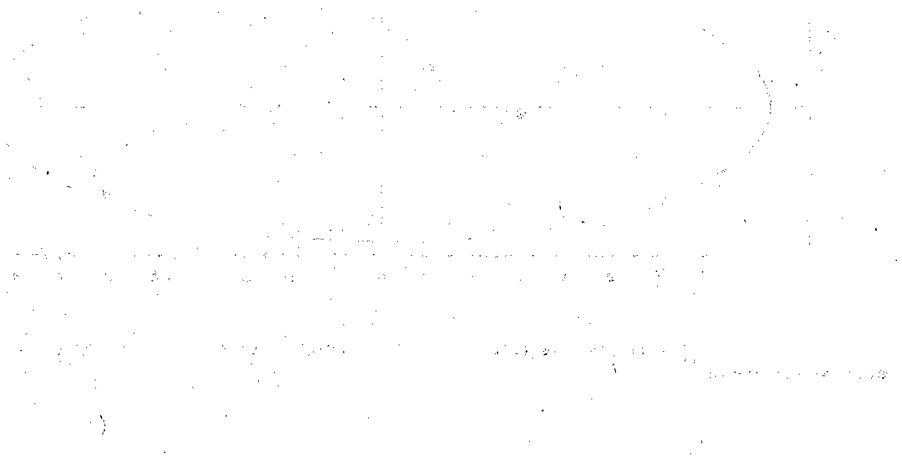


Figure 6-14: PLS DA scores plot of magic angle spinning

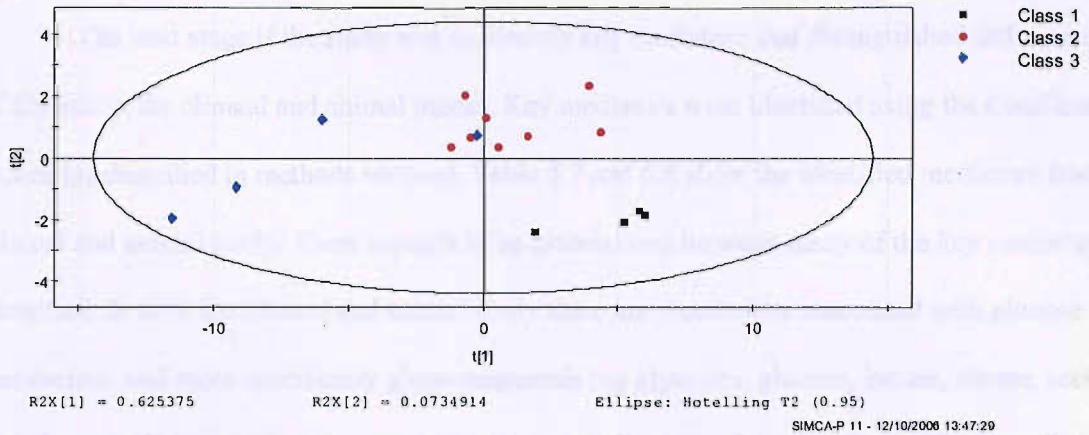


Figure 6-15: PLS DA scores plot of water extraction

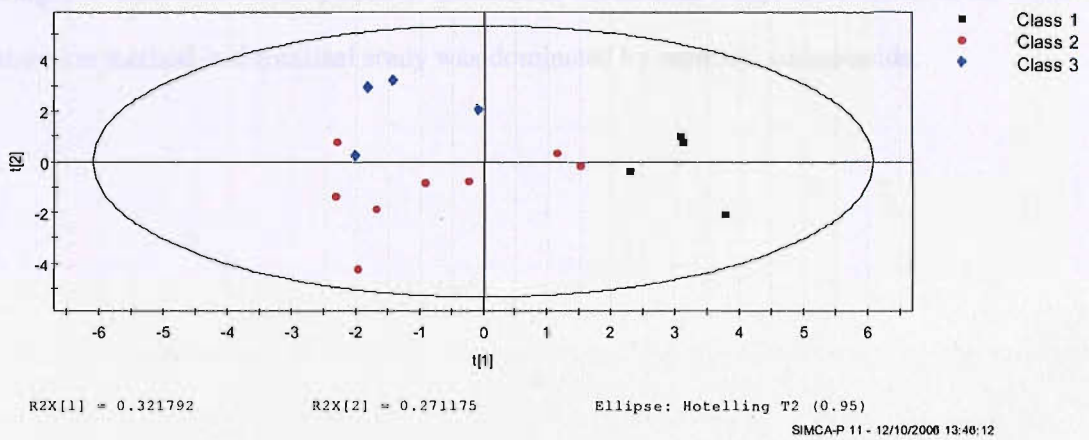
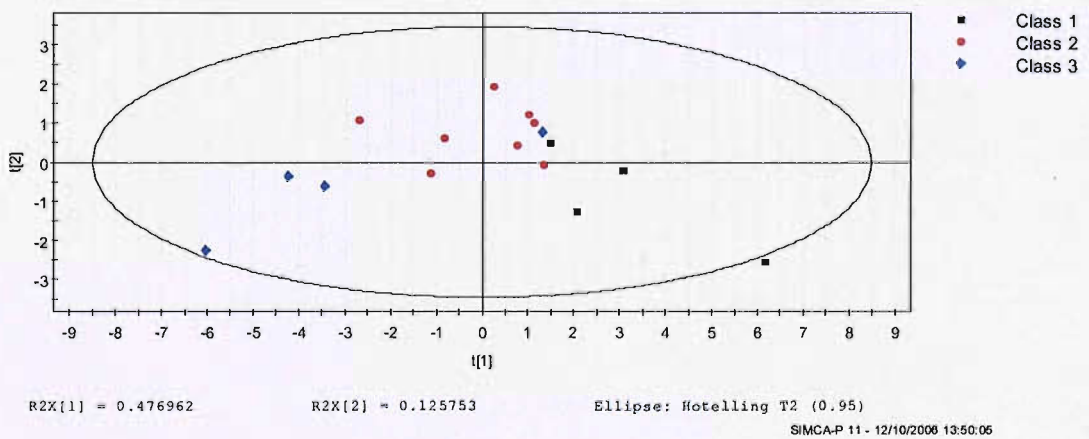


Figure 6-16: PLS DA scores plot of lipid extraction



Identification of key mediators which alter with progression of fibrosis

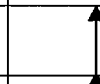
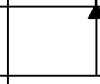
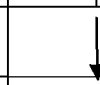
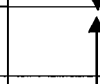
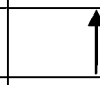
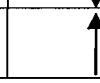
The next stage if the study was to identify key mediators that distinguished different stages of fibrosis in the clinical and animal model. Key mediators were identified using the Coefiicient CS values (as described in methods section). Table 6.7 and 6.8 show the identified mediators from the clinical and animal study. There appears to be associations between many of the key mediators identified. In both the clinical and animal study there are metabolites associated with glucose metabolism and more specifically gluconeogenesis (eg glycogen, glucose, lactate, citrate, acetate and alanine). There are also changes in choline or choline containing products in both studies. Some mediators also appear to be specific to either the clinical or animal study. For example changes in creatinine are only seen in the urine in the clinical study; in comparison the water extraction method in the animal study was dominated by essential amino-acids.

Table 6-7: Key mediators in clinical study

Putative Compound	Moderate fibrosis	Severe fibrosis	Univariate analysis
Urine			
3 hydroxybutyrate	↑	↑↑	P=0.031
Alanine	↓	↓↓	P=0.013
Lactate	↓	↓↓	P=0.011
Creatinine	↓	↓↓	P=0.004
Taurine	↑		P=0.069
Serum			
3 hydroxybutyrate	↑	↑↑	P=0.001
Alanine	↓	↓↓	P=0.047
Citrate		↑	P<0.001
Choline	↑		P=0.730
Acetate		↑	P=0.006

The arrows indicate the direction in which the metabolites contribute to the multivariate models. Single arrows represent changes relative to the "no fibrosis" model. Double arrows indicate alteration in signal of severe fibrosis relative to the moderate fibrosis group.

Table 6-8: Key mediators in animal study

	Early fibrosis	Cirrhosis	Univariate analysis
MAS analysis			
Glycogen			P=0.024
GPCHO/Cho			P=0.053
Choline			P=0.007
Water Extraction			
Choline			P=0.022
Glutamine			P=0.025
Glucose			P=0.003
Glutamate			P<0.001
Valine			P=0.018
Leucine/lysine			P=0.165
Lipid extraction			
Phosphatidyl choline			P=0.001
Glycerol			P=0.008

The arrows indicate the direction in which the metabolites contribute to the multivariate models. Single arrows represent changes relative to the "no fibrosis" model. Double arrows indicate alteration in signal of severe fibrosis relative to the moderate fibrosis group.

Translating the metabolic profile into a diagnostic algorithm for the clinical study

To compare the "potential" of key mediators found in this study as a diagnostic, performance was compared to a validated panel maker test of liver fibrosis, the ELF panel. A diagnostic algorithm was produced for the Metabonomic panel (described in the methods section) using logistic regression of the key identified mediators (shown in table 6.7) from the serum (alanine, lactate, citrate, 3 hydroxybutyrate and choline) and urine (alanine, creatinine, lactate, taurine and 3 hydroxybutyrate). The variables serum alanine and serum citrate remained significant ($p<0.1$) after removal of the other mediators. The metabonomic algorithm is shown below:

Metabonomic panel algorithm: 9.086 + 3.03 ln (serum citrate) – ln (serum alanine)

This algorithm was used to distinguish different stages of fibrosis and compared to the ELF panel with AUC values shown in table 6.9 below. The data suggests improving performance of both panels in distinguishing more severe forms of fibrosis, consistent with findings in the earlier part of the thesis. The very small numbers in this study are reflected by the wide standard of error. Also the metabonomic panel was derived on this cohort and therefore this represents a training set AUC in comparison to the ELF which is a validation set AUC. This analysis is exploratory and although the improvement in AUC statistics at distinguishing no fibrosis from the rest is encouraging, external validation is required on much larger numbers.

Table 6-9: Performance of metabonomic panel and ELF panel in distinguishing different stages of fibrosis

Panel test	No fibrosis versus the rest F0 vs F1/2/3/4	Mild fibrosis vs Moderate fibrosis F0/1 vs F2/3/4	Cirrhosis vs the rest F0/1/2/3 vs F4
ELF	0.76 +/- 0.07	0.84 +/- 0.07	0.95 +/- 0.03
Metabonomic Panel	0.86 +/- 0.05	0.85 +/- 0.06	0.94 +/- 0.04

6.3 Discussion

This study represents a metabonomic assessment of liver fibrosis in two distinct models of liver injury. A hypothesis free discovery approach was used to generate a pattern or "fingerprint" of metabolites that was not suspected prior to analysis. A pattern of metabolites was identified that appears to be associated with the presence of hepatic fibrosis. Furthermore, these signals appear to alter in early fibrosis and are associated with common metabolic pathways that are shared by both the clinical and animal models. Two very different models of liver fibrosis have been used to maximise the information obtained. This approach has significant power because the mechanisms and topography of injury are distinct, so the identification of metabolic pathways shared across the models are therefore more likely to be truly associated with fibrosis rather than being insult specific.

The identified pattern in this study, appear to relate to gluconeogenesis rather than components of matrix metabolism (the association of mediators is shown in figure 6.17 below). An increase in gluconeogenesis has previously been shown to occur in cirrhosis but the current study indicates that this pattern may occur in very early fibrosis [168-171]. The reduction in signal of alanine, in both the serum and urine, at an early stage of fibrosis is of relevance. Alanine is the primary amino-acid utilised for gluconeogenesis. Previous studies have demonstrated that patients with cirrhosis not only have reduced serum levels of alanine but also increased hepatic extraction as measured by catheterisation of the hepatic vasculature [170;172]. There is also evidence that this occurs in an animal model of hepatocellular carcinoma, with increased hepatic transport of alanine occurring in animals with tumour compared to controls[173].

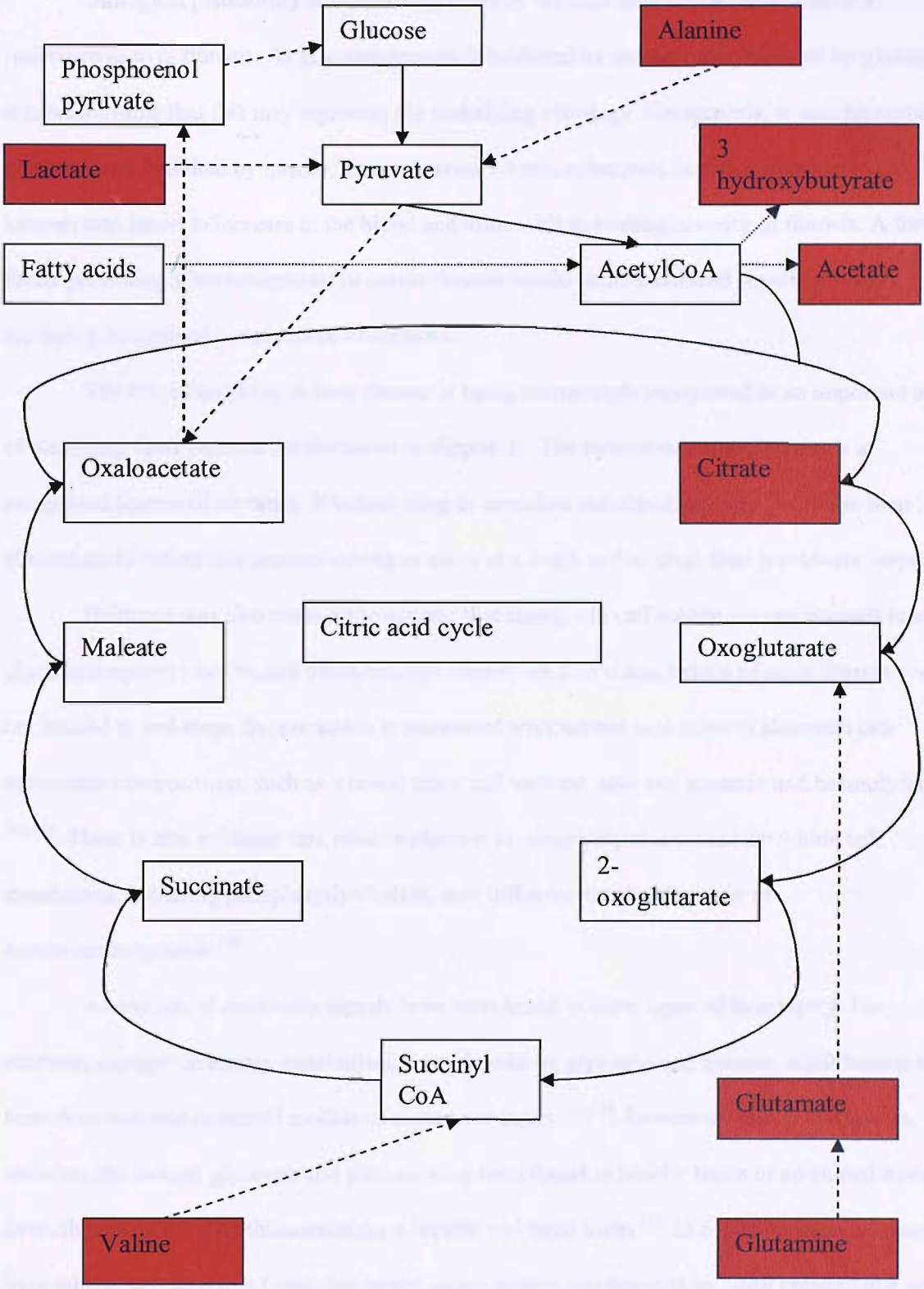


Figure 6-17: Glucose pathways highlighted by the study (identified mediators in red).

Biological plausibility is further suggested by the emerging importance of insulin resistance in liver fibrosis. As gluconeogenesis is inhibited by insulin (and promoted by glucagon) it is conceivable that this may represent the underlying aetiology. Ketogenesis, is also promoted by glucagon and inhibited by insulin, and of interest 3-hydroxybutyrate (a major liver generated ketone) was found to increase in the blood and urine with increasing severity of fibrosis. A further factor promoting gluconeogenesis in severe disease would include reduced hepatic glycogen storage, a recognised consequence of cirrhosis.

The role of nutrition in liver disease is being increasingly recognised as an important aspect of managing these patients (as discussed in chapter 1). The reduction in muscle mass is a recognised feature of cirrhosis. It is intriguing to speculate that the changes in creatinine seen in the clinical study reflect this process staring to occur at a much earlier stage than previously suspected.

Evidence was also obtained to suggest that changes in cell membrane components (choline, glycerophosphorylcholine and phosphatydylcholine) are also characteristic of early fibrosis and are not limited to end stage disease which is associated with indices indicative of abnormal cell membrane compositions, such as a raised mean cell volume, spur cell anaemia and haemolysis [174;175]. There is also evidence that relative changes in phospholipid composition within cell membranes, including phosphatydylcholine, may influence the development of hepatocarcinogenesis [176].

Alterations of metabolic signals have been found in other types of liver injury. For example, changes in energy metabolism, as evidenced by glycogen and glucose, disturbances have been demonstrated in animal models of acute liver injury [177;178]. Moreover, similar changes in amino-acids, lactate, glycogen and glucose have been found in hepatic tissue of an animal model of liver fibrosis induced by thioacteamide, a hepatic and renal toxin [179]. In a clinical study of acute liver failure, investigators found that raised serum glutamine (detected by NMR) predicted a poor outcome and hypothesised that this was related to excess ammonia [180]. Different types of liver injury may therefore share similar metabolic perturbations. While this may not be useful to distinguish aetiology it may have potential in measuring severity of injury.

The choice of the optimal biofluid in metabonomic studies is yet to be determined. Theoretically, urine would be the preferred option as it is less invasive than a blood sample. However studies have suggested greater inter and intra variation in urine samples compared to plasma samples [181]. The different dilutions of urine obtained from human participants and wider range of pH, in comparison to plasma, also pose technical difficulties. This is exemplified in this study by the fact that four urine samples were excluded because of being too dilute for analysis by ^{1}H- NMR. A mitigating factor of analysing urine is the spectral profile of plasma is influenced by broad bands from protein and liopoprotein signals in contrast to urine where low molecular weight metabolites produce a greater number of sharp lines. The identification of mediators from both urine and serum is therefore encouraging and differences in metabolites, between the biofluids, will be influenced by both biology and nuances of NMR spectroscopy.

Whilst this study has revealed a perturbed pattern of metabolites it has not produced definitive diagnostic biomarkers. The metabolic panel, comprising of alanine and citrate, produced in the final analysis is essentially an illustration of the potential of this technology as a diagnostic. The small numbers make any algorithm derived by logistic regression analysis extremely difficult to interpret. It is likely that if this analysis was performed on a larger sample that different key mediators would emerge in the final algorithm. This raises the interesting question of whether metabonomics could be used directly for the diagnosis of liver disease. On the positive side, the technology is powerful as highlighted by the ability to distinguish a breadth of compounds ranging from lipids to amino acids. Furthermore the ability to quantify these mediators is extremely valuable. Whist the capital outlay for NMR equipment is expensive the throughput can be rapid with the potential of processing hundreds of samples per day in an automated fashion. Thus central NMR facilities could service a large geographical area. On the negative side, the diagnostic strength lies in its ability to detect small differences in a range of mediators and thus will inevitably result in more complex tests, associated with the need for more advanced statistical tools in the analysis and interpretation of data. The greatest weakness in metabonomics perhaps lies in the influence of environmental factors such as diet, diurnal variation and medications on

metabonomics. Whilst one of the strengths of this study was to try and control for these factors, for a general diagnostic this would be extremely impractical.

An alternative application of metabonomics may be to highlight pathways that are important in disease. Exploration of these pathways may then allow the identification of biomarkers that can be detected by more "pragmatic" technologies such as ELISA. More importantly, this technology may reveal potential therapeutic targets and have the ability to detect early response to potential pharmacological intervention. A really exciting potential of metabonomics is in the area of prognostic modelling. If metabolic profiles can be created for liver disease, prognosis could be ascertained with greater certainty. For example, having a certain metabonomic profile may predispose to a very low risk of subsequent fibrosis and therefore the decision to treat may be delayed. The metabonomic profile could then be measured regularly and if the individual was found to be deviating away from this profile to another profile with a higher risk of fibrosis, the decision to treat reconsidered. In this way treatment and prognosis decisions would be individually tailored but based on models derived from the general "disease population". For this to be successful, large cohort data with hard clinical end points would need to be collected.

Designed to test proof of concept, this study has a number of limitations. It does not address cause and effect with respect to the identified metabolic pathways. Thus the changes could be a functional consequence of fibrosis or contribute to fibrosis. Furthermore, metabolic pathways such as gluconeogenesis are dynamic, and this study is measuring mediators at a cross sectional time point. To address these issues longitudinal studies would need to be performed. Parallel work in animal models would aid the understanding of pathophysiological mechanisms, particularly if key metabolites can be manipulated. The total numbers in the study are small and there is a danger of type 1 and type 2 statistical errors. Although internal validation of the models has been performed, external validation using independent data sets is required to substantiate the findings. The generalisability of the findings in this study is uncertain and the effect of diet, concurrent medical conditions and medication will need to be examined carefully. The absence of paired hepatic tissue and blood/urine from the same species makes comparisons between different biofluids more difficult. This was not undertaken because of cost implications for the study but will

need to be considered for future work. Finally, some of the key mediators remain unidentified and this will require further work to see if they are intermediates of the suspected pathways or represent new pathways.

In conclusion this is a small study but there appears to be a pattern of metabolites which alter at an early stage of fibrosis. Whilst this does not represent a definitive diagnostic tool it may serve as a method to highlight critical pathways. Exploration of these pathways may yield a) diagnostic markers that can be readily obtained by simple technology b) therapeutic targets that can be exploited for future anti fibrotic treatment.

Chapter 7 : Discussion

7.1 Findings and implications from the thesis

The systematic reviews highlighted the breadth of serum markers that are potentially available for the diagnosis of liver fibrosis. In NAFLD the majority of studies have concentrated on elucidating single variables associated with severe fibrosis. This is in contrast to CHC where the tests have evolved into panel marker tests with finalised diagnostic algorithms. A number of key issues emerged from the systematic reviews. Firstly, there the key end points of distinguishing stages of fibrosis tended to be moderate (CHC) or severe fibrosis (NAFLD). The performance of the markers was greatest at distinguishing or excluding severe disease. Secondly, some of the diagnostic statistics are limited in their translation into clinical practice. Thirdly, the dependence on the liver biopsy; an invasive and imperfect reference test for the development of non-invasive markers.

The systematic reviews provided a platform for the remaining thesis. The performance of serum markers was explored at different end points of fibrosis in NAFLD. Simple markers, identified in the systematic review, were combined with the ELF panel marker test and found to improve diagnostic performance. The clinical utility model was used to demonstrate how diagnostic tests could be used in current clinical practice.

The final part of the thesis explored how a technology platform, metabonomics, could aid the development of diagnostic markers in hepatitis C. In this novel, prospective study stages of fibrosis were associated with different metabolic profiles.

During the thesis common themes have arisen and these issues are discussed below in greater detail.

7.2 What are the key stages of fibrosis?

A fundamental issue of all diagnostic tests is what end points need to be distinguished? In liver fibrosis, the stages of fibrosis that are chosen often relate to treatment initiation points. This is exemplified by the diagnostic literature in chronic hepatitis C. At the start of this thesis, treatment guidelines for antiviral therapy were based on demonstrating moderate disease on liver biopsy [182]. The vast majority of studies in the HCV systematic review concentrated on distinguishing minimal fibrosis (Metavir stages F0/1) from moderate fibrosis (Metavir stages F2/3/4). The basis of this end point was a consensus statement made, in the context of treating patients with CHC, by the European Association of the study of Liver Disaese (EASL)[183]. During this thesis, national guidelines have changes because of strong evidence that treating early disease in CHC is cost effective [47]. Thus, within the context of CHC one might expect new diagnostic studies to emerge, concentrating on distinguishing no disease from any fibrosis.

The fact that the end points for diagnostic studies are based on treatment decisions does have some logic. Currently, there is no specific treatment for fibrosis per se and therefore management strategies are centred on treating the underlying insult and identifying severe disease for stratification into surveillance programmes. Furthermore, as the liver biopsy is an invasive test, it adds ethical considerations as to which patient should be recruited into potential diagnostic studies. However, there are some disadvantages with this process. Firstly, treatment options are dynamic. In NAFLD, there is no definitive treatment for the underlying condition but it is likely these will emerge in the near future. Secondly, even when treatment exists, guidelines will change with emerging evidence as illustrated above. This creates a potential lag time between treatment strategies and diagnostic tools to aid implementation. Finally, and perhaps more importantly, there are strong reasons why the diagnosis of liver fibrosis is important independent of treatment options. As discussed in chapter 1, the true prevalence of liver fibrosis and cirrhosis are unknown because of the asymptomatic nature of these conditions and the ethical considerations of using the liver

biopsy to ascertain epidemiological data. Having accurate "population" epidemiology will aid the planning of health care for the complications of liver disease.

7.3 Diagnostic tests and clinical utility

The thesis revealed a wide range of diagnostic statistics that are used to assess diagnostic tests. These range from sensitivity, specificity, predictive values, likelihood ratios, diagnostic odds ratios and receiver operator curves. The statistical merit of these parameters is not in doubt. However a recurrent theme throughout the thesis was the translation of these different statistics into clinical practice. The ROC statistic is the standard diagnostic test adopted by the majority of studies. It is a summary statistic and reflects the overall performance of the test across all thresholds. Whilst it produces a numerical value it lacks clinical "tangibility". Furthermore, it is unclear whether an increase of an AUC from 0.70 to 0.72, for example, confers pragmatic diagnostic benefit in clinical practice. The Clinical Utility model developed in chapter 3, in the context of the CHC systematic review, attempts to put some context of diagnostic tests within clinical practice. Clinicians use diagnostic tests to "rule in disease" or "rule out disease". The use of extreme thresholds to minimise false positives and false negatives gives the clinician confidence that they are not inappropriately intervening or denying intervention to the test population. In the context of liver fibrosis, currently this intervention may range from treatment (any fibrosis in CHC), life style advice (any fibrosis in NAFLD) or surveillance for the complications of liver disease (cirrhosis).

Choosing the acceptable "error rate" in liver fibrosis is more complicated than other diseases because firstly there is no specific treatment for fibrosis and secondly the implications of missing fibrosis may take years to manifest. In this thesis, conservative thresholds were chosen, to keep the error rate to 10 %. However this error rate may be inappropriately too high or low. Therefore how can one determine what error rate is acceptable? A national postal survey to all clinicians managing patients with liver fibrosis may have been one possible strategy to evaluated unacceptable "error rates". A major problem with this is the unpredictable response rates

associated with postal surveys. Framing the question and making it context specific can improve the quality of information gathered. However if a good response rate is not achieved there may be a danger of response bias and unrepresentative views. Whilst this may give information of the range of error rate it is difficult to know if this truly reflects practice of the majority. Alternatively, using a Delphi method is a method of obtaining consensus opinion. A panel of experts is approached with specific questions about a topic. Several rounds of iteration occur, in which experts are shown each others responses, with the final goal of obtaining consensus. If a wide range of experts are included the findings will have more weight but it is potentially more difficult to reach agreement. The difficulties of the process in this topic are illustrated by a recent Delphi survey amongst Italian gastroenterologists [184]. This survey addressed the question of the role of the liver biopsy in HCV. 108 expert opinions were invited to make judgements on 12 clinical scenarios. The first interesting observation from this survey was that the response rate was 57 % for the first round and fell to 36 % for the second round. Furthermore there was wide divergence of opinion about which patients should undergo liver biopsy in the scenarios not governed by treatment guidelines. Crucially this was not resolved after the final round of iteration.

Diagnostic tests are often used in a complex manner in clinical practice. In essence they are used to increase or decrease the pre-test probability of having a certain condition. The pre-test probability is largely based on the prevalence of disease in that clinical setting. However other diagnostic tools, such as patient history, clinical examination, baseline blood tests and radiology, will also affect the pre test probability of any subsequent test. The influence of the initial diagnostic tools will vary depending on the clinician and the setting. An experienced hepatologist, with access to specialist radiology in a liver centre, may have different requirements form a non-invasive serum test compared to a junior clinician in a district general hospital. Thus although the majority of clinicians will choose thresholds near the extremes of the test rather than the middle, there will be some variability on the precise threshold and the associated error rate. Therefore presenting a range of error rates for diagnostic tests could maintain clinical applicability whilst retaining flexibility. One method of trying to distinguish the spectrum of error rates would be to have focussed workshops with different users of a potential diagnostic test (hepatologists, gastroenterologists,

primary care physicans and junior clinicians). Furthermore, by incorporating the other parameters (clinical signs, simple biochemistry, haematology and radiology) into clinical scenarios a better understanding of the contribution of non-invasive markers could be made.

7.4 The liver biopsy

The liver biopsy is a valuable diagnostic tool. It provides a breadth of information which is important in ascertaining disease aetiology, pathophysiological mechanisms of disease and duration of injury. Non-invasive markers cannot provide this range of information and thus the liver biopsy will always have a role in the diagnosis of liver disease. For the specific purpose of assessing liver fibrosis there are limitations which have recurred during this thesis. Firstly, it is an invasive test. This has a major influence on the design of diagnostic studies as previously discussed as there is probable verification bias. Secondly, there is uncertainty about how accurately information from the biopsy truly reflects fibrosis in the liver because of a) sampling error and b) variation in the interpretation of liver biopsies within and between pathologists and c) the constraints of using a categorical scoring system for measuring a dynamic, continuous process.

The length of biopsy has been shown to be an important factor for the assessment of liver fibrosis. Bedossa et al showed using image analysis that fibrosis heterogeneity had a coefficient of variation in the following lengths of liver biopsy: 55 % for 15 mm, 45 % in 25 mm and 30 % in 100 mm [87]. In the same study classification could be improved by increasing the liver biopsy from 15 mm (65 % correct classification) to 25 mm (75 % correct classification). One solution would be to prospectively recruit participants for diagnostic studies with large biopsies (>25 mm). This is more difficult in practice because many centres now obtain biopsies under radiological guidance and there is a perceived risk that larger biopsies are associated with a greater number of complications (there is no published evidence to support this perception). An alternative strategy has been to differentiate biopsies on size using a methodology called discordant analysis [185;186]. Biopsies that are "inadequate", smaller than 10 mm in length or contain less than 5 portal tracts, are classified as discordant if not in agreement with the non-invasive biochemical test. If there is a

known cause for biochemical failure, eg haemolysis of the sample, this is classified as biochemical failure but any remaining discordance is classed as a biopsy failure. The obvious limitation with this analysis is that if a biopsy is below 10 mm, it may have an increased probability of being inaccurate but cannot be definitively assumed to be. Furthermore, if the small biopsy below 10mm and non-invasive marker are in agreement (i.e. concordant) there is the possibility that they are both wrong. Simply excluding very small biopsies may not be a solution as there is evidence that length of the biopsy decreases (associated with an increased number of fragments) with increasing disease severity [187]; hence a danger of selection bias.

The quantification of fibrosis using scoring systems presents a number of problems. Many of these scoring systems were developed for assessing response to treatment in the context of chronic hepatitis C (eg Metavir, scheuer, Ishak and knodell) and have subsequently been adapted for other diseases as well as being adopted for diagnostic studies. The scores are semi quantitative descriptors but non-invasive markers are often continuous scores. To address this discrepancy there have been work looking at quantifying fibrosis using automated morphometry [188;189]. Liver biopsies are scored for the quantity of fibrosis using digital analysers and computer packages. There are some technical issues with analysers being very sensitive to the staining material used and variation of readings between the same liver core of tissue. Moreover whilst the quantity of fibrosis detected at morphometry correlates to the stages of fibrosis, importantly there is not a liner relationship. For example the progression from no fibrosis (Ishak stage 0) to bridging fibrosis (Ishak stage 4) to cirrhosis (Ishak stage 6) is associated with fibrosis measurements of 1.9 %, 13.7 %, and 27.8 % respectively [190]. This suggests that not only is the quantity of fibrosis important for staging of fibrosis but also the distribution of fibrosis, architectural disturbance and vascular involvement. Thus in the example above, the progression form stage 0 to stage 4 in Ishak may only be associated with an increase in approximately 12 % of fibrosis but the connection of fibrotic tissue between the portal tract and central vein (with the associated angiogenesis and subsequent shunting of blood) is what determines functional and prognostic information. As technology continues to develop, incorporating topography and quantity of fibrosis, it may increase the value of this automated technology. Importantly, sampling bias of the biopsy will still remain an issue.

Therefore if the liver biopsy has limitations as a reference standard what alternative reference standards exist? Severity of fibrosis on liver biopsy has been shown to predict progression to cirrhosis [84]. Recently Neal at al presented evidence that liver fibrosis is an independent predictor of liver related mortality in a long term cohort of approximately 3,000 patients with CHC [191]. Therefore as histology is a surrogate of clinical outcome measures there is logic to measure performance of non-invasive markers against clinical outcome directly. An issue with planning and performing these "direct" diagnostic studies is the complications of liver disease take time to develop and therefore they need to be conducted over a period of time. Nonetheless such studies are starting to emerge. Poynard et al examined the Fibrotest at baseline in predicting clinical outcomes at 5 years. They showed that the AUC of fibrotest was 0.96 for predicting death (AUC of biopsy 0.87) and 0.96 for the complications of disease (AUC of biopsy 0.91)[192].

7.5 Biological plausibility of serum markers

Serum markers are thought to be measures (directly or indirectly) of structure or function. Some of the non-invasive markers have direct functional relevance. Albumin and platelets, which emerged as simple markers in the NAFLD systematic review, are markers of hepatic function and it is intuitive they are associated with severe fibrosis. The ELF panel constituents, hyaluronic acid, TIMP 1 and PIIINP are thought of as direct markers of matrix deposition and thus a measure of structure. However they may be measuring both structure and function. For example, within cirrhosis some patients will have active disease with increased matrix deposition but others will have relatively inactive disease. The ELF panel has an extremely high diagnostic accuracy for the detection of the majority of cases with cirrhosis. It is conceivable that within the patients with "inactive disease" matrix production is still high, but net matrix deposition is minimal because of equivalent matrix degradation. However, it is intriguing to speculate these markers may also reflect the functional consequences of cirrhosis because of reduced extraction of the markers due to intrahepatic shunting secondary to "capillarisation" of the sinusoids.

The potential to measure functional parameters of the liver is of enormous relevance as this will be an important determinant of prognosis. A suggestion from the metabonomics study is that this technology is measuring a functional signature of the liver. Moreover, a unifying theme from the NAFLD and HCV parts of the thesis is the association of insulin resistance and liver fibrosis. A cause of this insulin resistance in HCV may be related to the virus itself, in particular genotype 3 [193], or the underlying metabolic determinants [194]. Additionally, there is in vitro evidence that hyperinsulinaemia directly stimulates hepatic stellate cell to upregulate mediators of fibrosis [195]. However could the presence of fibrosis per se accentuate insulin resistance? It is well recognised that liver cirrhosis can precede the development of insulin resistance in a variety of aetiologies [196]. The mechanisms of this remain to be determined but there is interest in the role of insulin like growth factors (IGFs). IGFs have been shown to a) counteract the effects of insulin resistance and b) reduce insulin secretion [197;198]. The major source of this hormone is from hepatocytes[199] and lower levels of IGF-1 have been found in advanced fibrosis and cirrhosis[200;201]. Therefore a conceivable hypothesis is that insulin resistance and liver fibrosis propagate one another. This may be one explanation of why measures of insulin resistance are common in non-invasive markers of liver disease; they may signify both the aetiology and consequence of fibrosis. Carefully designed, prospective studies are needed to resolve the issue of biological cause and effect but the lack of this knowledge does not necessarily detract from using these measures as a diagnostic.

7.6 Strengths and limitations of the thesis

The strengths of the thesis lay in the breadth of work that was undertaken in exploring diagnostic tests in liver fibrosis. This included a detailed examination of the literature and then using these findings to influence the planning and analysis of the subsequent primary research. The exploration of different models of fibrosis enabled common aspects of fibrosis to be highlighted whilst also identifying nuances of individual disease aetiologies. A wide range of statistical analyses have been employed, and attempts have been made to give statistical measures context in

clinical practice. Using two very different diagnostic tests, serum panel markers and metabonomics, has focussed the relevant advantages and disadvantages of not only the technologies but also the conceptual idea of hypothesis driven versus hypothesis generating research.

Two major aetiological diseases were chosen to exemplify the diagnosis of liver fibrosis, HCV and NAFLD. A limitation of this thesis is other aetiologies of liver disease have not been studied, in particular alcohol, because of time constraints. There are similarities between alcohol and non-alcoholic liver disease in the histological distribution of fibrosis. The treatment for alcoholic liver disease is abstinence and therefore a spectrum of end-points that clinicians wish to measure may be similar to NAFLD eg any fibrosis (using the diagnostic as a therapeutic) or severe fibrosis (for surveillance of complications). However there are also important caveats within alcoholic liver disease. Distinguishing alcoholic hepatitis is of particular relevance because of the existence of pharmacological treatment for this entity. It is noticeable the diagnostic literature in alcohol concentrates on both non-invasive markers for the histological complications of alcoholic liver disease (steatosis, necroinflammation and fibrosis) in addition to prognostic models centred on alcoholic hepatitis per se [202;203]. Thus whilst broad findings about diagnostic tests may be applicable to all aetiologies of liver disease, there are idiosyncrasies within individual diseases which makes extrapolation more difficult.

This thesis did not examine the role of non-invasive markers for the diagnosis of necroinflammation and steatosis in NAFLD and HCV. Inflammation and fibrosis are linked by similar immunological pathways and therefore it is likely that common markers will exist for these separate histological conditions. Moreover, it has been shown that necroinflammation on the index liver biopsy in HCV predicts future liver fibrosis [204]. Some diagnostic studies have developed non-invasive markers for a score combining necroinflammation and fibrosis [101]. However this implies fibrosis with no inflammation which is unintuitive. Separate non-invasive may therefore be needed for clinically relevant pathological entities and there needs to be flexibility of which stages of disease are diagnosed, not only within fibrosis but also between fibrosis, inflammation and steatosis.

In the HCV and NAFLD studies novel diagnostic algorithms were produced. However the relatively small sample size from which they were derived and absence of external validation limits the assessment of their diagnostic potential. The strict inclusion and exclusion criteria in the metabonomics study, whilst important for reasons detailed earlier, limits the generalisability of the findings.

7.7 Improving non-invasive tests and further work

There are a number of ways in which diagnostic tests can be improved. Combining existing serum marker panels or using them in a step wise fashion is one possibility. Sebastiani et al combined two panel tests in a serial manner, APRI test on all subjects and then Fibrotest on any "unclassified" patients [205]. They suggest that the number of liver biopsies using this method can be reduced by 50 % whilst retaining 94 % accuracy (using fibrotest alone they calculated 43 % of liver biopsies could be avoided). The benefit of combining algorithms (7 % of avoided biopsies) is obviously balanced by any additional cost and increased complexity.

The use of imaging to assess fibrosis has many attractions. It potentially allows the whole organ to be sampled, thus avoiding many of the sampling error issues. Traditional imaging using, as discussed in the introduction, has been disappointing for the diagnosis of liver fibrosis. Novel techniques have started to emerge and show promise. Microbubble ultrasound, assesses hepatic vein transit time and there is evidence that this changes in clinically relevant stages of fibrosis [206]. Transient elastography (TE) is a method of generating an elastic shear wave across the liver; reliant on the hypothesis that increasing fibrosis reduces elasticity of the liver so that in more severe disease there is increased "stiffness" resulting in greater velocity of the propagated wave. There have been studies in a range of disease aetiologies [207]. There are issues of reproducibility and technical difficulties, for example an increased BMI has implications for the acquisition of data using TE. However the potential to combine imaging techniques and serum may be fruitful particularly if they are measuring different aspects of structure and function. Recently TE has been

combined with a serum panel marker test in CHC with an AUC of 0.88 for detecting moderate fibrosis [208]. Further validation of combined imaging and serum marker tests are awaited.

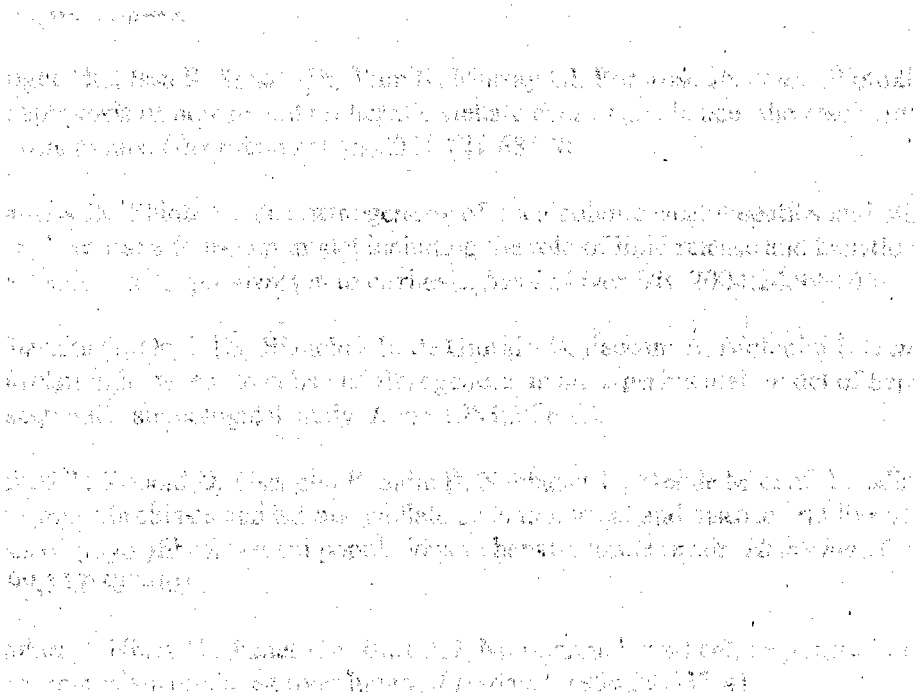
Using novel technology platforms is a powerful mechanism for exploring liver fibrosis, as illustrated by metabonomics. There have been studies in genomics [209], proteomics [210] and glycomics [211] which have created diagnostic algorithms for liver fibrosis. An issue with these technologies, as discussed with metabonomics, are the practical and cost implications of these tests. However having a range of diagnostic tests, including the liver biopsy, will give clinicians and researchers a greater number of tools depending on the context of the test. A simple, reproducible, cheap test may an attractive option in the community to screen for liver fibrosis but a combination of tests, including imaging, biopsy and "omics" may be required to assess suitability and response to potential antifibrotic agents.

The initial aims of any further work would be to validate the findings from the thesis in independent cohorts. Testing the algorithms created in the NAFLD study on cohorts with a spectrum of fibrosis in differing clinical settings would also be valuable. The metabonomics study has generated a number of hypotheses which need to be tested. Are changes in glucose metabolism a result of fibrosis, causing fibrosis or both? Will pharmacological intervention reverse these changes and can metabonomics detect these changes at an earlier stage than serum markers or histology? Can metabonomics be translated into more pragmatic diagnostic tests? The mechanistic questions will require longitudinal studies and parallel work in animal and human models. To determine the practical and generalisability issues further studies enrolling broader groups of patients with fewer restrictions, on environmental factors such as diet and medication, are required.

The limitations of the liver biopsy suggest using an alternative reference standard. As histology is simply a surrogate for clinical outcome measures, the obvious solution is to compare biomarkers directly with clinical outcome measures. The lag time between the complications of disease and fibrosis will be an issue. Initial studies could concentrate on severe disease, i.e. enrol patients with cirrhosis only and then follow up with serial measurement of biomarkers until complications ensue.

7.8 Overall conclusions

The diagnosis of liver fibrosis using non-invasive biofluids is important for epidemiological, prognostic, therapeutic, pragmatic and economical reasons. Currently, serum non-invasive markers are at different stages in NAFLD and CHC but are gradually evolving into panel marker tests demonstrating current clinical utility. Metabonomics is an exciting technology, offering an alternative approach, and further work is needed to verify if this can be translated into diagnostic benefit or aid therapeutic development. Continuing to improve the accuracy and spectrum of diagnosis of non-invasive markers will increase the application of these tests in routine clinical practice.



Reference List

1. Chief Medical Officer. Liver cirrhosis starting to strike at a younger age. 2001. Ref Type: Report

2. Hepatitis C in England- the first Health Protection Agency Report (2005). 2005. Ref Type: Report

3. Neuschwander-Tetri BA,.Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003;**37**:1202-19.

4. Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology* 2000;**32**:477-81.

5. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT *et al.* Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am.J.Gastroenterol.* 2002;**97**:2614-8.

6. Canbay A, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 2004;**39**:273-8.

7. Canbay A, Taimr P, Torok N, Higuchi H, Friedman S, Gores GJ. Apoptotic body engulfment by a human stellate cell line is profibrogenic. *Lab Invest* 2003;**83**:655-63.

8. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S *et al.* Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J.Clin.Invest* 1998;**102**:538-49.

9. Wright MC, Issa R, Smart DE, Trim N, Murray GI, Primrose JN *et al.* Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. *Gastroenterology* 2001;**121**:685-98.

10. Wanless IR,.Shiota K. The pathogenesis of nonalcoholic steatohepatitis and other fatty liver diseases: a four-step model including the role of lipid release and hepatic venular obstruction in the progression to cirrhosis. *Semin.Liver Dis.* 2004;**24**:99-106.

11. Ballardini G, Degli ES, Bianchi FB, de Giorgi LB, Faccani A, Biolchini L *et al.* Correlation between Ito cells and fibrogenesis in an experimental model of hepatic fibrosis. A sequential stereological study. *Liver* 1983;**3**:58-63.

12. Knittel T, Kobold D, Piscaglia F, Saile B, Neubauer K, Mehde M *et al.* Localization of liver myofibroblasts and hepatic stellate cells in normal and diseased rat livers: distinct roles of (myo-)fibroblast subpopulations in hepatic tissue repair. *Histochem.Cell Biol.* 1999;**112**:387-401.

13. Mathew J, Hines JE, James OF, Burt AD. Non-parenchymal cell responses in paracetamol (acetaminophen)-induced liver injury. *J.Hepatol.* 1994;**20**:537-41.

14. Shi Z, Wakil AE, Rockey DC. Strain-specific differences in mouse hepatic wound healing are mediated by divergent T helper cytokine responses. *Proc.Natl.Acad.Sci.U.S.A* 1997;**94**:10663-8.

15. Forbes SJ, Russo FP, Rey V, Burra P, Rugge M, Wright NA *et al.* A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology* 2004;**126**:955-63.

16. Rockey DC. Hepatic blood flow regulation by stellate cells in normal and injured liver. *Semin.Liver Dis.* 2001;**21**:337-49.

17. Corpechot C, Barbu V, Wendum D, Kinnman N, Rey C, Poupon R *et al.* Hypoxia-induced VEGF and collagen I expressions are associated with angiogenesis and fibrogenesis in experimental cirrhosis. *Hepatology* 2002;**35**:1010-21.

18. Rosmorduc O, Wendum D, Corpechot C, Galy B, Sebbagh N, Raleigh J *et al.* Hepatocellular hypoxia-induced vascular endothelial growth factor expression and angiogenesis in experimental biliary cirrhosis. *Am.J.Pathol.* 1999;**155**:1065-73.

19. Okuda K, Ohnishi K, Kimura K, Matsutani S, Sumida M, Goto N *et al.* Incidence of portal vein thrombosis in liver cirrhosis. An angiographic study in 708 patients. *Gastroenterology* 1985;**89**:279-86.

20. Belli L, Romani F, Sansalone CV, Aseni P, Rondinara G. Portal thrombosis in cirrhotics. A retrospective analysis. *Ann.Surg.* 1986;**203**:286-91.

21. Gaiani S, Bolondi L, Li BS, Zironi G, Siringo S, Barbara L. Prevalence of spontaneous hepatofugal portal flow in liver cirrhosis. Clinical and endoscopic correlation in 228 patients. *Gastroenterology* 1991;**100**:160-7.

22. Wanless IR, Wong F, Blendis LM, Greig P, Heathcote EJ, Levy G. Hepatic and portal vein thrombosis in cirrhosis: possible role in development of parenchymal extinction and portal hypertension. *Hepatology* 1995;**21**:1238-47.

23. Sato Y, Asada Y, Hara S, Marutsuka K, Tamura K, Hayashi T *et al.* Hepatic stellate cells (Ito cells) in veno-occlusive disease of the liver after allogeneic bone marrow transplantation. *Histopathology* 1999;**34**:66-70.

24. El Youssef M, Mu Y, Huang L, Stellmach V, Crawford SE. Increased expression of transforming growth factor-beta1 and thrombospondin-1 in congenital hepatic fibrosis: possible role of the hepatic stellate cell. *J.Pediatr.Gastroenterol.Nutr.* 1999;**28**:386-92.

25. Ryder SD, Irving WL, Jones DA, Neal KR, Underwood JC, Trent Hepatitis CSG. Progression of hepatic fibrosis in patients with hepatitis C: a prospective repeat liver biopsy study.[see comment]. *Gut.53(3):451-5,* 2004.

26. Fontaine H, Nalpas B, Poulet B, Carnot F, Zylberberg H, Brechot C *et al.* Hepatitis activity index is a key factor in determining the natural history of chronic hepatitis C. *Hum.Pathol.* 2001;**32**:904-9.

27. Lindh M, Horal P, Dhillon AP, Norkrans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histological activity in chronic hepatitis B. *J.Viral Hepat.* 2000;**7**:258-67.

28. Merican I, Guan R, Amarapuka D, Alexander MJ, Chutaputti A, Chien RN *et al.* Chronic hepatitis B virus infection in Asian countries. *J.Gastroenterol.Hepatol.* 2000;**15**:1356-61.

29. Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology.30(6):1356-62,* 1999.

30. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997;**349**:825-32.

31. Powell EE, Edwards-Smith CJ, Hay JL, Clouston AD, Crawford DH, Shorthouse C *et al.* Host genetic factors influence disease progression in chronic hepatitis C. *Hepatology.31(4):828-33,* 2000.

32. Wright M, Goldin R, Hellier S, Knapp S, Frodsham A, Hennig B *et al.* Factor V Leiden polymorphism and the rate of fibrosis development in chronic hepatitis C virus infection. *Gut* 2003;**52**:1206-10.

33. Dufour JF, DeLellis R, Kaplan MM. Regression of hepatic fibrosis in hepatitis C with long-term interferon treatment. *Dig.Dis.Sci.* 1998;**43**:2573-6.

34. Dufour JF, DeLellis R, Kaplan MM. Reversibility of hepatic fibrosis in autoimmune hepatitis. *Ann.Intern.Med.* 1997;**127**:981-5.

35. Shiratori Y, Imazeki F, Moriyama M, Yano M, Arakawa Y, Yokosuka O *et al.* Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann.Intern.Med.* 2000;**132**:517-24.

36. Hammel P, Couvelard A, O'Toole D, Ratouis A, Sauvanet A, Flejou JF *et al.* Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. *N.Engl.J.Med.* 2001;**344**:418-23.

37. Powell LW,.Kerr JF. Reversal of "cirrhosis" in idiopathic haemochromatosis following long-term intensive venesection therapy. *Australas.Ann.Med.* 1970;**19**:54-7.

38. Desmet VJ,.Roskams T. Cirrhosis reversal: a duel between dogma and myth. *J.Hepatol.* 2004;**40**:860-7.

39. Anthony PP, Ishak KG, Nayak NC, Poulsen HE, Scheuer PJ, Sobin LH. The morphology of cirrhosis. Recommendations on definition, nomenclature, and classification by a working group sponsored by the World Health Organization. *J.Clin.Pathol.* 1978;**31**:395-414.

40. World Health Organisation Statistical Information System; http://www.who.int/whosis/en/. 2004.
Ref Type: Report

41. Bosetti C, Levi F, Lucchini F, Zatonski WA, Negri E, La Vecchia C. Worldwide mortality from cirrhosis: an update to 2002. *J.Hepatol.* 2007;**46**:827-39.

42. Leon DA,.McCambridge J. Liver cirrhosis mortality rates in Britain from 1950 to 2002: an analysis of routine data. *Lancet* 2006;**367**:52-6.

43. Graudal N, Leth P, Marbjerg L, Galloe AM. Characteristics of cirrhosis undiagnosed during life: a comparative analysis of 73 undiagnosed cases and 149 diagnosed cases of cirrhosis, detected in 4929 consecutive autopsies. *J.Intern.Med.* 1991;**230**:165-71.

44. World health organisation. Weekly epidemiological record. 2002.
Ref Type: Report

45. Law MG, Dore GJ, Bath N, Thompson S, Crofts N, Dolan K *et al.* Modelling hepatitis C virus incidence, prevalence and long-term sequelae in Australia, 2001. *Int.J.Epidemiol.* 2003;**32**:717-24.

46. Zou S, Tepper M, El Saadany S. Prediction of hepatitis C burden in Canada. *Can.J.Gastroenterol.* 2000;**14**:575-80.

47. Grieve R, Roberts J, Wright M, Sweeting M, DeAngelis D, Rosenberg W *et al.* Cost effectiveness of interferon alpha or peginterferon alpha with ribavirin for histologically mild chronic hepatitis C. *Gut* 2006;**55**:1332-8.

48. Caldwell SH, Oelsner DH, Iezzoni JC, Hespenheide EE, Battle EH, Driscoll CJ. Cryptogenic cirrhosis: clinical characterization and risk factors for underlying disease. *Hepatology* 1999;**29**:664-9.

49. Poonawala A, Nair SP, Thuluvath PJ. Prevalence of obesity and diabetes in patients with cryptogenic cirrhosis: a case-control study. *Hepatology* 2000;**32**:689-92.

50. Sakugawa H, Nakasone H, Nakayoshi T, Kawakami Y, Yamashiro T, Maeshiro T *et al.* Clinical characteristics of patients with cryptogenic liver cirrhosis in Okinawa, Japan. *Hepatogastroenterology* 2003;**50**:2005-8.

51. Hui JM, Hodge A, Farrell GC, Kench JG, Kriketos A, George J. Beyond insulin resistance in NASH: TNF-alpha or adiponectin?[see comment]. *Hepatology.40(1):46-54,* 2004.

52. Leclercq IA, Farrell GC, Schriemer R, Robertson GR. Leptin is essential for the hepatic fibrogenic response to chronic liver injury. *J.Hepatol.* 2002;**37**:206-13.

53. Chitturi S, Farrell G, Frost L, Kriketos A, Lin R, Liddle C *et al.* Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: A manifestation of lipotoxicity? *Hepatology.Vol.36(2)()(pp 403-409), 2002.* 2002;403-9.

54. Prediction of the first variceal hemorrhage in patients with cirrhosis of the liver and esophageal varices. A prospective multicenter study. The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices. *N.Engl.J.Med.* 1988;**319**:983-9.

55. D'Amico G, Pagliaro L, Bosch J. The treatment of portal hypertension: a meta-analytic review. *Hepatology* 1995;**22**:332-54.

56. Moore KP, Wong F, Gines P, Bernardi M, Ochs A, Salerno F *et al.* The management of ascites in cirrhosis: report on the consensus conference of the International Ascites Club. *Hepatology* 2003;**38**:258-66.

57. Schenk P, Schoniger-Hekele M, Fuhrmann V, Madl C, Silberhumer G, Muller C. Prognostic significance of the hepatopulmonary syndrome in patients with cirrhosis. *Gastroenterology* 2003;**125**:1042-52.

58. Fallon MB, Abrams GA, Luo B, Hou Z, Dai J, Ku DD. The role of endothelial nitric oxide synthase in the pathogenesis of a rat model of hepatopulmonary syndrome. *Gastroenterology* 1997;**113**:606-14.

59. Nunes H, Lebrec D, Mazmanian M, Capron F, Heller J, Tazi KA *et al.* Role of nitric oxide in hepatopulmonary syndrome in cirrhotic rats. *Am.J.Respir.Crit Care Med.* 2001;**164**:879-85.

60. Taille C, Cadranel J, Bellocq A, Thabut G, Soubrane O, Durand F *et al.* Liver transplantation for hepatopulmonary syndrome: a ten-year experience in Paris, France. *Transplantation* 2003;**75**:1482-9.

61. Swanson KL, Wiesner RH, Krowka MJ. Natural history of hepatopulmonary syndrome: Impact of liver transplantation. *Hepatology* 2005;**41**:1122-9.

62. Lee SS. Cardiac abnormalities in liver cirrhosis. *West J.Med.* 1989;**151**:530-5.

63. Liu H, Song D, Lee SS. Cirrhotic cardiomyopathy. *Gastroenterol.Clin.Biol.* 2002;**26**:842-7.

64. Myers RP,.Lee SS. Cirrhotic cardiomyopathy and liver transplantation. *Liver Transpl.* 2000;**6**:S44-S52.

65. Velazquez RF, Rodriguez M, Navascues CA, Linares A, Perez R, Sotorrios NG *et al.* Prospective analysis of risk factors for hepatocellular carcinoma in patients with liver cirrhosis. *Hepatology* 2003;**37**:520-7.

66. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T *et al.* Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N.Engl.J.Med.* 1993;**328**:1797-801.

67. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004;**127**:S35-S50.

68. Park SZ, Nagorney DM, Czaja AJ. Hepatocellular carcinoma in autoimmune hepatitis. *Dig.Dis.Sci.* 2000;**45**:1944-8.

69. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004;**127**:S35-S50.

70. Quero JC,.Schalm SW. Subclinical hepatic encephalopathy. *Semin.Liver Dis.* 1996;**16**:321-8.

71. Nutritional status in cirrhosis. Italian Multicentre Cooperative Project on Nutrition in Liver Cirrhosis. *J.Hepatol.* 1994;**21**:317-25.

72. Alberino F, Gatta A, Amodio P, Merkel C, Di Pascoli L, Boffo G *et al.* Nutrition and survival in patients with liver cirrhosis. *Nutrition* 2001;**17**:445-50.

73. Alvares-da-Silva MR,.Reverbel dS. Comparison between handgrip strength, subjective global assessment, and prognostic nutritional index in assessing malnutrition and predicting clinical outcome in cirrhotic outpatients. *Nutrition* 2005;**21**:113-7.

74. Navasa M, Fernandez J, Rodes J. Bacterial infections in liver cirrhosis. *Ital.J.Gastroenterol.Hepatol.* 1999;**31**:616-25.

75. Borzio M, Salerno F, Piantoni L, Cazzaniga M, Angeli P, Bissoli F *et al.* Bacterial infection in patients with advanced cirrhosis: a multicentre prospective study. *Dig.Liver Dis.* 2001;**33**:41-8.

76. Caly WR,.Strauss E. A prospective study of bacterial infections in patients with cirrhosis. *J.Hepatol.* 1993;**18**:353-8.

77. Cirera I, Bauer TM, Navasa M, Vila J, Grande L, Taura P *et al.* Bacterial translocation of enteric organisms in patients with cirrhosis. *J.Hepatol.* 2001;**34**:32-7.

78. Hassner A, Kletter Y, Jedvab M, Aronson M, Shibolet S. Impaired monocyte function in liver cirrhosis. *Lancet* 1979;**1**:329-30.

79. Rajkovic IA,.Williams R. Abnormalities of neutrophil phagocytosis, intracellular killing and metabolic activity in alcoholic cirrhosis and hepatitis. *Hepatology* 1986;**6**:252-62.

80. Runyon BA, Morrissey RL, Hoefs JC, Wyle FA. Opsonic activity of human ascitic fluid: a potentially important protective mechanism against spontaneous bacterial peritonitis. *Hepatology* 1985;**5**:634-7.

81. Ellis G, Goldberg DM, Spooner RJ, Ward AM. Serum enzyme tests in diseases of the liver and biliary tree. *Am.J.Clin.Pathol.* 1978;**70**:248-58.

82. Afdhal NH,.Nunes D. Evaluation of liver fibrosis: a concise review. *Am.J.Gastroenterol.* 2004;**99**:1160-74.

83. Ryder SD, Irving WL, Jones DA, Neal KR, Underwood JC, Trent Hepatitis CSG. Progression of hepatic fibrosis in patients with hepatitis C: a prospective repeat liver biopsy study.[see comment]. *Gut.53(3):451-5,* 2004.

84. Yano M, Kumada H, Kage M, Ikeda K, Shimamatsu K, Inoue O *et al.* The long-term pathological evolution of chronic hepatitis C. *Hepatology* 1996;**23**:1334-40.

85. Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology* 2000;**32**:477-81.

86. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT *et al.* Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am.J.Gastroenterol.* 2002;**97**:2614-8.

87. Bedossa P, Dargere D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology* 2003;**38**:1449-57.

88. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. The French METAVIR Cooperative Study Group. *Hepatology* 1994;**20**:15-20.

89. Gandon Y, Olivie D, Guyader D, Aube C, Oberti F, Sebille V *et al.* Non-invasive assessment of hepatic iron stores by MRI. *Lancet* 2004;**363**:357-62.

90. Macdonald GA,.Peduto AJ. Magnetic resonance imaging (MRI) and diseases of the liver and biliary tract. Part 1. Basic principles, MRI in the assessment of diffuse and focal hepatic disease. *J.Gastroenterol.Hepatol.* 2000;**15**:980-91.

91. NICE. Peginterfon alpha and ribavavrin for the treatment of mild chronic hepatitis C. 2006.
Ref Type: Report

92. Gebo KA, Herlong HF, Torbenson MS, Jenckes MW, Chander G, Ghanem KG *et al.* Role of liver biopsy in management of chronic hepatitis C: a systematic review. *Hepatology* 2002;**36**:S161-S172.

93. Deeks JJ. Systematic reviews in health care: Systematic reviews of evaluations of diagnostic and screening tests. *BMJ* 2001;**323**:157-62.

94. Haynes RB,.Wilczynski NL. Optimal search strategies for retrieving scientifically strong studies of diagnosis from Medline: analytical survey. *BMJ* 2004;**328**:1040.

95. Whiting P, Rutjes AW, Reitsma JB, Bossuyt PM, Kleijnen J. The development of QUADAS: a tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. *BMC Med.Res.Methodol.* 2003;**3**:25.

96. Deeks J. Evaluations of diagnostic and screening tests. In Davey Smith G. AD, ed. *Systematic Reviews in Health Care*, 2006.

97. Gebo KA, Herlong HF, Torbenson MS, Jenckes MW, Chander G, Ghanem KG *et al.* Role of liver biopsy in management of chronic hepatitis C: a systematic review. *Hepatology* 2002;**36**:S161-S172.

98. Poynard T, Imbert-Bismut F, Munteanu M, Messous D, Myers RP, Thabut D *et al.* Overview of the diagnostic value of biochemical markers of liver fibrosis (FibroTest, HCV FibroSure) and necrosis (ActiTest) in patients with chronic hepatitis C. *Comp Hepatol.* 2004;**3**:8.

99. Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001;**357**:1069-75.

100. Rossi E, Adams L, Prins A, Bulsara M, de Boer B, Garas G *et al.* Validation of the FibroTest biochemical markers score in assessing liver fibrosis in hepatitis C patients. *Clin.Chem.* 2003;**49**:450-4.

101. Poynard T, McHutchison J, Manns M, Myers RP, Albrecht J. Biochemical surrogate markers of liver fibrosis and activity in a randomized trial of peginterferon alfa-2b and ribavirin. *Hepatology* 2003;**38**:481-92.

102. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS *et al.* A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003;**38**:518-26.

103. Le Calvez S, Thabut D, Messous D, Munteanu M, Ratziu V, Imbert-Bismut F *et al.* The predictive value of Fibrotest vs. APRI for the diagnosis of fibrosis in chronic hepatitis C. *Hepatology* 2004;**39**:862-3.

104. Forns X, Ampurdanes S, Llovet JM, Aponte J, Quinto L, Martinez-Bauer E *et al.* Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. *Hepatology* 2002;**36**:986-92.

105. Thabut D, Simon M, Myers RP, Messous D, Thibault V, Imbert-Bismut F *et al.* Noninvasive prediction of fibrosis in patients with chronic hepatitis C. *Hepatology* 2003;**37**:1220-1.

106. Sud A, Hui JM, Farrell GC, Bandara P, Kench JG, Fung C *et al.* Improved prediction of fibrosis in chronic hepatitis C using measures of insulin resistance in a probability index. *Hepatology* 2004;**39**:1239-47.

107. Leroy V, Monier F, Bottari S, Trocme C, Sturm N, Hilleret MN *et al.* Circulating matrix metalloproteinases 1, 2, 9 and their inhibitors TIMP-1 and TIMP-2 as serum markers of liver fibrosis in patients with chronic hepatitis C: comparison with PIIINP and hyaluronic acid. *Am.J.Gastroenterol.* 2004;**99**:271-9.

108. el Shorbagy E, Afefy AF, Ibrahem IA, Mangoud AM, Eissa MH, Sabee EI *et al.* Non-invasive markers and predictors of severity of hepatic fibrosis in HCV patients at Sharkia Governorate, Egypt. *J.Egypt.Soc.Parasitol.* 2004;**34**:459-78.

109. Patel K, Gordon SC, Jacobson I, Hezode C, Oh E, Smith KM *et al.* Evaluation of a panel of non-invasive serum markers to differentiate mild from moderate-to-advanced liver fibrosis in chronic hepatitis C patients. *J.Hepatol.* 2004;**41**:935-42.

110. Rosenberg WM, Voelker M, Thiel R, Becka M, Burt A, Schuppan D *et al.* Serum markers detect the presence of liver fibrosis: a cohort study. *Gastroenterology* 2004;**127**:1704-13.

111. Kaul V, Friedenberg FK, Braitman LE, Anis U, Zaeri N, Fazili J *et al.* Development and validation of a model to diagnose cirrhosis in patients with hepatitis C. *Am.J.Gastroenterol.* 2002;**97**:2623-8.

112. Fortunato G, Castaldo G, Oriani G, Cerini R, Intrieri M, Molinaro E *et al.* Multivariate discriminant function based on six biochemical markers in blood can predict the cirrhotic evolution of chronic hepatitis. *Clin.Chem.* 2001;**47**:1696-700.

113. Deeks JJ. Systematic reviews in health care: Systematic reviews of evaluations of diagnostic and screening tests. *BMJ* 2001;**323**:157-62.

114. Hanley JA,.McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 1982;**143**:29-36.

115. Deeks JJ. Systematic reviews in health care: Systematic reviews of evaluations of diagnostic and screening tests. *BMJ* 2001;**323**:157-62.

116. Greenhalgh T. How to read a paper. Papers that report diagnostic or screening tests. *BMJ* 1997;**315**:540-3.

117. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM *et al.* The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. *Ann.Intern.Med.* 2003;**138**:W1-12.

118. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM *et al.* Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Standards for Reporting of Diagnostic Accuracy. *Clin.Chem.* 2003;**49**:1-6.

119. Deeks JJ. Systematic reviews in health care: Systematic reviews of evaluations of diagnostic and screening tests. *BMJ* 2001;**323**:157-62.

120. Sterne JA, Gavaghan D, Egger M. Publication and related bias in meta-analysis: power of statistical tests and prevalence in the literature. *J.Clin.Epidemiol.* 2000;**53**:1119-29.

121. Colloredo G, Guido M, Sonzogni A, Leandro G. Impact of liver biopsy size on histological evaluation of chronic viral hepatitis: the smaller the sample, the milder the disease. *J.Hepatol.* 2003;**39**:239-44.

122. Poynard T, Munteanu M, Imbert-Bismut F, Charlotte F, Thabut D, Le Calvez S *et al.* Prospective analysis of discordant results between biochemical markers and biopsy in patients with chronic hepatitis C.[see comment]. *Clinical Chemistry.50(8):1344-55,* 2004.

123. Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A *et al.* The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005;**129**:113-21.

124. Adams LA, Sanderson S, Lindor KD, Angulo P. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J.Hepatol.* 2005;**42**:132-8.

125. Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology.30(6):1356-62,* 1999.

126. Sakugawa H, Nakayoshi T, Kobashigawa K, Yamashiro T, Maeshiro T, Miyagi S *et al.* Clinical usefulness of biochemical markers of liver fibrosis in patients with nonalcoholic fatty liver disease. *World Journal of Gastroenterology.11(2):255-9,* 2005.

127. Albano E, Mottaran E, Vidali M, Reale E, Saksena S, Occhino G *et al.* Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis. *Gut.Vol.54(7)()(pp 987-993), 2005.* 2005;987-93.

128. Mofrad P, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA *et al.* Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology* 2003;**37**:1286-92.

129. Shimada M, Hashimoto E, Kaneda H, Noguchi S, Hayashi N. Nonalcoholic steatohepatitis: Risk factors for liver fibrosis. *Hepatology Research.Vol.24(4)()(pp 429-438), 2002.Date of Publication: 01 DEC 2002.* 2002;429-38.

130. Dixon JB, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese.[see comment]. *Gastroenterology.121(1):91-100,* 2001.

131. Beymer C, Kowdley KV, Larson A, Edmonson P, Dellinger EP, Flum DR. Prevalence and predictors of asymptomatic liver disease in patients undergoing gastric bypass surgery. *Archives of Surgery.138(11):1240-4,* 2003.

132. Bugianesi E, Manzini P, D'Antico S, Vanni E, Longo F, Leone N *et al.* Relative contribution of iron burden, HFE mutations, and insulin resistance to fibrosis in nonalcoholic fatty liver.[see comment]. *Hepatology.39(1):179-87,* 2004.

133. Dixon JB, Bhathal PS, Jonsson JR, Dixon AF, Powell EE, O'Brien PE. Pro-fibrotic polymorphisms predictive of advanced liver fibrosis in the severely obese. *Journal of Hepatology.39(6):967-71,* 2003.

134. Hui JM, Hodge A, Farrell GC, Kench JG, Kriketos A, George J. Beyond insulin resistance in NASH: TNF-alpha or adiponectin?[see comment]. *Hepatology.40(1):46-54,* 2004.

135. Guidorizzi de Siqueira AC, Cotrim HP, Rocha R, Carvalho FM, de Freitas LA, Barreto D *et al.* Non-alcoholic fatty liver disease and insulin resistance: importance of risk factors and histological spectrum. *Eur.J.Gastroenterol.Hepatol.* 2005;**17**:837-41.

136. Suzuki A, Angulo P, Lymp J, Li D, Satomura S, Lindor K. Hyaluronic acid, an accurate serum marker for severe hepatic fibrosis in patients with non-alcoholic fatty liver disease. *Liver Int.* 2005;**25**:779-86.

137. Angulo P, Alba LM, Petrovic LM, Adams LA, Lindor KD, Jensen MD. Leptin, insulin resistance, and liver fibrosis in human nonalcoholic fatty liver disease. *J.Hepatol.* 2004;**41**:943-9.

138. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R *et al.* Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 2003;**37**:917-23.

139. Hashimoto E, Yatsuji S, Kaneda H, Yoshioka Y, Taniai M, Tokushige K *et al.* The characteristics and natural history of Japanese patients with nonalcoholic fatty liver disease. *Hepatol.Res.* 2005.

140. Ong JP, Elariny H, Collantes R, Younoszai A, Chandhoke V, Reines HD *et al.* Predictors of nonalcoholic steatohepatitis and advanced fibrosis in morbidly obese patients. *Obesity Surgery.Vol.15(3)()(pp 310-315), 2005.* 2005;310-5.

141. de L, V, Combes M, Trouette H, Winnock M, Amouretti M, de Mascarel A *et al.* Should a liver biopsy be done in patients with subclinical chronically elevated transaminases? *European Journal of Gastroenterology & Hepatology.16(9):879-83,* 2004.

142. Ratziu V, Giral P, Charlotte F, Bruckert E, Thibault V, Theodorou I *et al.* Liver fibrosis in overweight patients. *Gastroenterology* 2000;**118**:1117-23.

143. Sorrentino P, Tarantino G, Conca P, Perrella A, Terracciano ML, Vecchione R *et al.* Silent non-alcoholic fatty liver disease - A clinical-histological study. *Journal of Hepatology.Vol.41(5)()(pp 751-757), 2004.* 2004;751-7.

144. Crespo J, Fernandez-Gil P, Hernandez-Guerra M, Cayon A, Mayorga M, Dominguez-Diez A *et al.* Are there predictive factors of severe liver fibrosis in morbidly obese patients with non-alcoholic steatohepatitis? *Obesity Surgery.11(3):254-7,* 2001.

145. Fierbinteanu-Braticevici C, Bengus A, Neamtu M, Usvat R. The risk factors of fibrosis in nonalcoholic steatohepatitis. *Romanian Journal of Internal Medicine.40(1-4):81-8,* 2002.

146. Loguercio C, De Simone T, D'Auria MV, de S, I, Federico A, Tuccillo C *et al.* Non-alcoholic fatty liver disease: a multicentre clinical study by the Italian Association for the Study of the Liver. *Digestive & Liver Disease.36(6):398-405,* 2004.

147. Santos VN, Leite-Mor MM, Kondo M, Martins JR, Nader H, Lanzoni VP *et al.* Serum laminin, type IV collagen and hyaluronan as fibrosis markers in non-alcoholic fatty liver disease. *Braz.J.Med.Biol.Res.* 2005;**38**:747-53.

148. Yesilova Z, Yaman H, Oktenli C, Ozcan A, Uygun A, Cakir E *et al.* Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic Fatty liver disease. *American Journal of Gastroenterology.100(4):850-5,* 2005.

149. Koruk M, Savas MC, Yilmaz O, Taysi S, Karakok M, Gundogdu C *et al.* Serum lipids, lipoproteins and apolipoproteins levels in patients with nonalcoholic steatohepatitis. *Journal of Clinical Gastroenterology.37(2):177-82,* 2003.

150. Hartleb M, Kajor M, Gupinska-Kajor M, Nowak A, Rudzki K, Waluga M *et al.* Nonalcoholic fatty liver disease; who does not benefit from hepatic biopsy? *Gastroenterologia Polska.Vol.12(3)()(pp 209-213), 2005.* 2005;209-13.

151. Chitturi S, Farrell G, Frost L, Kriketos A, Lin R, Fung C *et al.* Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: a manifestation of lipotoxicity?[erratum appears in Hepatology 2002 Nov;36(5):1307]. *Hepatology.36(2):403-9,* 2002.

152. Brunt EM, Neuschwander-Tetri BA, Oliver D, Wehmeier KR, Bacon BR. Nonalcoholic steatohepatitis: histologic features and clinical correlations with 30 blinded biopsy specimens. *Human Pathology.35(9):1070-82,* 2004.

153. Paradis V, Perlemuter G, Bonvoust F, Dargere D, Parfait B, Vidaud M *et al.* High glucose and hyperinsulinemia stimulate connective tissue growth factor expression: a potential mechanism involved in progression to fibrosis in nonalcoholic steatohepatitis. *Hepatology* 2001;**34**:738-44.

154. Samuel VT, Liu ZX, Qu X, Elder BD, Bilz S, Befroy D *et al.* Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J.Biol.Chem.* 2004;**279**:32345-53.

155. Zeng MD, Lu LG, Mao YM, Qiu DK, Li JQ, Wan MB *et al.* Prediction of significant fibrosis in HBeAg-positive patients with chronic hepatitis B by a noninvasive model. *Hepatology* 2005;**42**:1437-45.

156. Guechot J, Laudat A, Loria A, Serfaty L, Poupon R, Giboudeau J. Diagnostic accuracy of hyaluronan and type III procollagen amino-terminal peptide serum assays as markers of liver fibrosis in chronic viral hepatitis C evaluated by ROC curve analysis. *Clin.Chem.* 1996;**42**:558-63.

157. Pares A, Deulofeu R, Gimenez A, Caballeria L, Bruguera M, Caballeria J *et al.* Serum hyaluronate reflects hepatic fibrogenesis in alcoholic liver disease and is useful as a marker of fibrosis. *Hepatology* 1996;**24**:1399-403.

158. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;**41**:1313-21.

159. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT *et al.* Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am.J.Gastroenterol.* 2002;**97**:2614-8.

160. Lindon JC, Holmes E, Bollard ME, Stanley EG, Nicholson JK. Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers* 2004;**9**:1-31.

161. Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW *et al.* Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabonomics. *Nat.Med.* 2002;**8**:1439-44.

162. Odunsi K, Wollman RM, Ambrosone CB, Hutson A, McCann SE, Tammela J *et al.* Detection of epithelial ovarian cancer using 1H-NMR-based metabonomics. *Int.J.Cancer* 2005;**113**:782-8.

163. Yang J, Xu G, Zheng Y, Kong H, Pang T, Lv S *et al.* Diagnosis of liver cancer using HPLC-based metabonomics avoiding false-positive result from hepatitis and

hepatocirrhosis diseases. *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.* 2004;**813**:59-65.

164. Yang J, Zhao X, Liu X, Wang C, Gao P, Wang J *et al.* High performance liquid chromatography-mass spectrometry for metabonomics: potential biomarkers for acute deterioration of liver function in chronic hepatitis B. *J.Proteome.Res.* 2006;**5**:554-61.

165. Maggini C,.Raballo A. Exploring depression in schizophrenia. *Eur.Psychiatry* 2006;**21**:227-32.

166. Issa R, Zhou X, Constandinou CM, Fallowfield J, Millward-Sadler H, Gaca MD *et al.* Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. *Gastroenterology* 2004;**126**:1795-808.

167. Plummer JL, Ossowicz CJ, Whibley C, Ilsley AH, Hall PD. Influence of intestinal flora on the development of fibrosis and cirrhosis in a rat model. *J.Gastroenterol.Hepatol.* 2000;**15**:1307-11.

168. Changani KK, Jalan R, Cox IJ, Ala-Korpela M, Bhakoo K, Taylor-Robinson SD *et al.* Evidence for altered hepatic gluconeogenesis in patients with cirrhosis using in vivo 31-phosphorus magnetic resonance spectroscopy. *Gut* 2001;**49**:557-64.

169. Petersen KF, Krssak M, Navarro V, Chandramouli V, Hundal R, Schumann WC *et al.* Contributions of net hepatic glycogenolysis and gluconeogenesis to glucose production in cirrhosis. *Am.J.Physiol* 1999;**276**:E529-E535.

170. Piniewska DM, McCulloch AJ, Bramble MG, Taylor R, Record CO, Alberti KG. Glucose turnover in compensated hepatic cirrhosis. *Horm.Metab Res.* 1986;**18**:834-7.

171. Krahenbuhl L, Lang C, Ludes S, Seiler C, Schafer M, Zimmermann A *et al.* Reduced hepatic glycogen stores in patients with liver cirrhosis. *Liver Int.* 2003;**23**:101-9.

172. Owen OE, Reichle FA, Mozzoli MA, Kreulen T, Patel MS, Elfenbein IB *et al.* Hepatic, gut, and renal substrate flux rates in patients with hepatic cirrhosis. *J.Clin.Invest* 1981;**68**:240-52.

173. Bink H, Carbo N, Felipe A, Lopez-Soriano FJ, Argiles JM. Hepatic transport of gluconeogenic substrates during tumor growth in the rat. *Cancer Invest* 2001;**19**:248-55.

174. Morse EE. Mechanisms of hemolysis in liver disease. *Ann.Clin.Lab Sci.* 1990;**20**:169-74.

175. Salvioli G, Rioli G, Lugli R, Salati R. Membrane lipid composition of red blood cells in liver disease: regression of spur cell anaemia after infusion of polyunsaturated phosphatidylcholine. *Gut* 1978;**19**:844-50.

176. Abel S, Smuts CM, de Villiers C, Gelderblom WC. Changes in essential fatty acid patterns associated with normal liver regeneration and the progression of hepatocyte nodules in rat hepatocarcinogenesis. *Carcinogenesis* 2001;**22**:795-804.

177. Atherton HJ, Bailey NJ, Zhang W, Taylor J, Major H, Shockcor J *et al.* A combined 1H-NMR spectroscopy- and mass spectrometry-based metabolomic study of the PPAR-alpha null mutant mouse defines profound systemic changes in metabolism linked to the metabolic syndrome. *Physiol Genomics* 2006;**27**:178-86.

178. Waters NJ, Waterfield CJ, Farrant RD, Holmes E, Nicholson JK. Metabonomic deconvolution of embedded toxicity: application to thioacetamide hepato- and nephrotoxicity. *Chem.Res.Toxicol.* 2005;**18**:639-54.

179. Constantinou MA, Theocharis SE, Mikros E. Application of metabonomics on an experimental model of fibrosis and cirrhosis induced by thioacetamide in rats. *Toxicol.Appl.Pharmacol.* 2007;**218**:11-9.

180. Saxena V, Gupta A, Nagana Gowda GA, Saxena R, Yachha SK, Khetrapal CL. 1H NMR spectroscopy for the prediction of therapeutic outcome in patients with fulminant hepatic failure. *NMR Biomed.* 2006;**19**:521-6.

181. Lenz EM, Bright J, Wilson ID, Morgan SR, Nash AF. A 1H NMR-based metabonomic study of urine and plasma samples obtained from healthy human subjects. *J.Pharm.Biomed.Anal.* 2003;**33**:1103-15.

182. NICE. Interferon alpha (pegylated and non-pegylated) and ribavarin for the treatment of chronic hepatitis C. 1-1-2004.
Ref Type: Report

183. EASL International Consensus Conference on Hepatitis C. Paris, 26-28, February 1999, Consensus Statement. European Association for the Study of the Liver. *J.Hepatol.* 1999;**30**:956-61.

184. Almasio PL, Niero M, Angioli D, Ascione A, Gullini S, Minoli G *et al.* Experts' opinions on the role of liver biopsy in HCV infection: a Delphi survey by the Italian Association of Hospital Gastroenterologists (A.I.G.O.). *J.Hepatol.* 2005;**43**:381-7.

185. Poynard T, Munteanu M, Imbert-Bismut F, Charlotte F, Thabut D, Le Calvez S *et al.* Prospective analysis of discordant results between biochemical markers and biopsy in patients with chronic hepatitis C. *Clin.Chem.* 2004;**50**:1344-55.

186. Halfon P, Bourliere M, Deydier R, Botta-Fridlund D, Renou C, Tran A *et al.* Independent prospective multicenter validation of biochemical markers (fibrotest-actitest) for the prediction of liver fibrosis and activity in patients with chronic hepatitis C: the fibropaca study. *Am.J.Gastroenterol.* 2006;**101**:547-55.

187. Malik AH, Kumar KS, Malet PF, Jain R, Prasad P, Ostapowicz G. Correlation of percutaneous liver biopsy fragmentation with the degree of fibrosis. *Aliment.Pharmacol.Ther.* 2004;**19**:545-9.

188. Wright M, Thursz M, Pullen R, Thomas H, Goldin R. Quantitative versus morphological assessment of liver fibrosis: semi-quantitative scores are more robust than digital image fibrosis area estimation. *Liver Int.* 2003;**23**:28-34.

189. O'Brien MJ, Keating NM, Elderiny S, Cerda S, Keaveny AP, Afdhal NH *et al.* An assessment of digital image analysis to measure fibrosis in liver biopsy specimens of patients with chronic hepatitis C. *Am.J.Clin.Pathol.* 2000;**114**:712-8.

190. Standish RA, Cholongitas E, Dhillon A, Burroughs AK, Dhillon AP. An appraisal of the histopathological assessment of liver fibrosis. *Gut* 2006;**55**:569-78.

191. Neal K,.Irving WL. Excess mortality rates in a cohort of patients infected with the hepatitis C virus: a prospective study. *Gut* 2007.

192. Ngo Y, Munteanu M, Messous D, Charlotte F, Imbert-Bismut F, Thabut D *et al.* A prospective analysis of the prognostic value of biomarkers (FibroTest) in patients with chronic hepatitis C. *Clin.Chem.* 2006;**52**:1887-96.

193. Hui JM, Sud A, Farrell GC, Bandara P, Byth K, Kench JG *et al.* Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]. *Gastroenterology* 2003;**125**:1695-704.

194. Fartoux L, Poujol-Robert A, Guechot J, Wendum D, Poupon R, Serfaty L. Insulin resistance is a cause of steatosis and fibrosis progression in chronic hepatitis C. *Gut* 2005;**54**:1003-8.

195. Paradis V, Perlemuter G, Bonvoust F, Dargere D, Parfait B, Vidaud M *et al.* High glucose and hyperinsulinemia stimulate connective tissue growth factor expression: a potential mechanism involved in progression to fibrosis in nonalcoholic steatohepatitis. *Hepatology* 2001;**34**:738-44.

196. Petrides AS, Vogt C, Schulze-Berge D, Matthews D, Strohmeyer G. Pathogenesis of glucose intolerance and diabetes mellitus in cirrhosis. *Hepatology* 1994;**19**:616-27.

197. Le Roith D. Seminars in medicine of the Beth Israel Deaconess Medical Center. Insulin-like growth factors. *N.Engl.J.Med.* 1997;**336**:633-40.

198. Conchillo M, Prieto J, Quiroga J. Insulin-like growth factor I (IGF-I) and liver cirrhosis. *Rev.Esp.Enferm.Dig.* 2007;**99**:156-64.

199. Daughaday WH,.Rotwein P. Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr.Rev.* 1989;**10**:68-91.

200. Shmueli E, Miell JP, Stewart M, Alberti KG, Record CO. High insulin-like growth factor binding protein 1 levels in cirrhosis: link with insulin resistance. *Hepatology* 1996;**24**:127-33.

201. Lorenzo-Zuniga V, Bartoli R, Masnou H, Montoliu S, Morillas RM, Planas R. Serum Concentrations of Insulin-Like Growth Factor-I (IGF-I) as a Marker of Liver Fibrosis in Patients With Chronic Hepatitis C. *Dig.Dis.Sci.* 2007.

202. Maddrey WC, Boitnott JK, Bedine MS, Weber FL, Jr., Mezey E, White RI, Jr. Corticosteroid therapy of alcoholic hepatitis. *Gastroenterology* 1978;**75**:193-9.

203. Forrest EH, Evans CD, Stewart S, Phillips M, Oo YH, McAvoy NC *et al.* Analysis of factors predictive of mortality in alcoholic hepatitis and derivation and validation of the Glasgow alcoholic hepatitis score. *Gut* 2005;**54**:1174-9.

204. Ryder SD, Irving WL, Jones DA, Neal KR, Underwood JC. Progression of hepatic fibrosis in patients with hepatitis C: a prospective repeat liver biopsy study. *Gut* 2004;**53**:451-5.

205. Sebastiani G, Vario A, Guido M, Noventa F, Plebani M, Pistis R *et al.* Stepwise combination algorithms of non-invasive markers to diagnose significant fibrosis in chronic hepatitis C. *J.Hepatol.* 2006;**44**:686-93.

206. Lim AK, Taylor-Robinson SD, Patel N, Eckersley RJ, Goldin RD, Hamilton G *et al.* Hepatic vein transit times using a microbubble agent can predict disease severity non-invasively in patients with hepatitis C. *Gut* 2005;**54**:128-33.

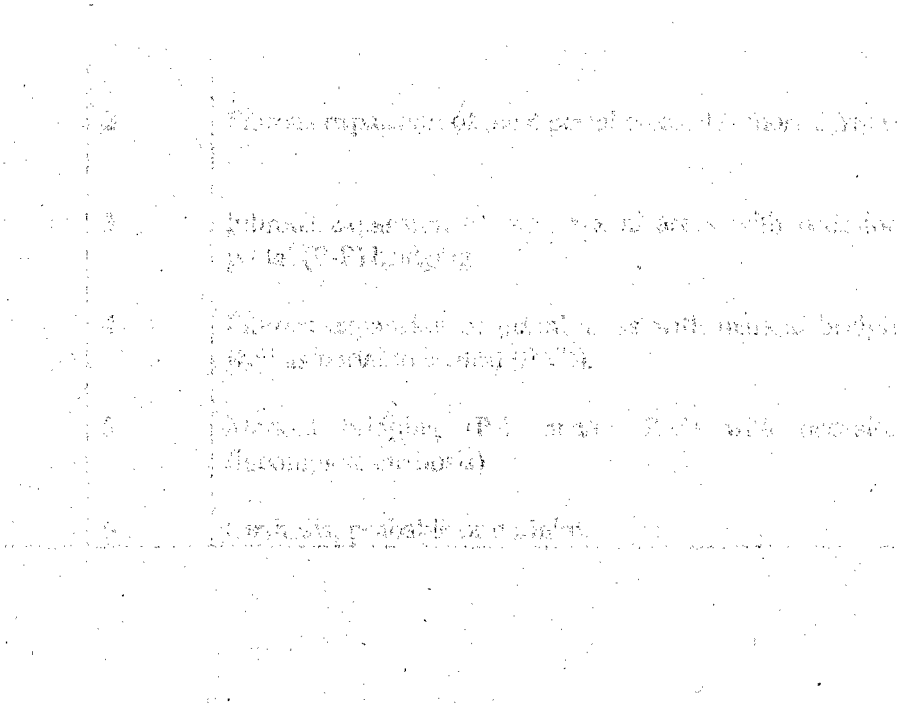
207. Foucher J, Chanteloup E, Vergniol J, Castera L, Le Bail B, Adhoute X *et al.* Diagnosis of cirrhosis by transient elastography (FibroScan): a prospective study. *Gut* 2006;**55**:403-8.

208. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M *et al.* Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005;**128**:343-50.

209. Huang H, Shiffman ML, Cheung RC, Layden TJ, Friedman S, Abar OT *et al.* Identification of two gene variants associated with risk of advanced fibrosis in patients with chronic hepatitis C. *Gastroenterology* 2006;**130**:1679-87.

210. Poon TC, Hui AY, Chan HL, Ang IL, Chow SM, Wong N *et al.* Prediction of liver fibrosis and cirrhosis in chronic hepatitis B infection by serum proteomic fingerprinting: a pilot study. *Clin.Chem.* 2005;**51**:328-35.

211. Callewaert N, Van Vlierberghe H, Van Hecke A, Laroy W, Delanghe J, Contreras R. Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics. *Nat.Med.* 2004;**10**:429-34.



Appendix 1 : staging systems for classification of fibrosis

SCORING SYSTEM	STAGE	DESCRIPTION
METAVIR	0	No fibrosis.
	1	Stellate enlargement of portal tracts but without septa formation.
	2	Enlargement of portal tracts with rare septa formation.
	3	Numerous septa without cirrhosis.
	4	Cirrhosis.
SCHEUER	0	None.
	1	Enlarged, fibrotic portal tracts.
	2	Periportal or portal-portal septa but intact architecture.
	3	Fibrosis with architectural distortion but no obvious cirrhosis.
	4	Probable or definite cirrhosis.
ISHAK	0	No fibrosis.
	1	Fibrous expansion of some portal areas, +/- short fibrous septa.
	2	Fibrous expansion of most portal areas, +/- short fibrous septa.
	3	Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging.
	4	Fibrous expansion of portal areas with marked bridging ((P-P) as well as portal to central (P-C)).
	5	Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis).
	6	Cirrhosis, probable or definite.

Appendix 2 : Medline search strategy for HCV SR

1. serum markers.mp. or exp Biological Markers/

2. limit 1 to (human and english language and yr=1990)

3. YKL 40.mp.

4. exp LAMININ/

5. (MMP-2 or TIMP 1).mp.

6. PIIINP.mp.

7. hyaluron$.mp.

8. (MMP$ or TIMP$ or type$ collagen).mp. [mp=title, original title, abstract, name of substance, mesh subject heading]

9. (tenascin or $globulin).mp. [mp=title, original title, abstract, name of substance, mesh subject heading]

10. non-invasive marker.mp.

11. 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10

12. aspartate transaminase.mp. [mp=title, original title, abstract, name of substance, mesh subject heading]

13. alanine transferase.mp. [mp=title, original title, abstract, name of substance, mesh subject heading]

14. aminotransferase.mp. [mp=title, original title, abstract, name of substance, mesh subject heading]

15. (ALT or AST).mp. [mp=title, original title, abstract, name of substance, mesh subject heading]

16. liver fibrosis marker.mp. [mp=title, original title, abstract, name of substance, mesh subject heading]

17. 12 or 13 or 14 or 15 or 16

18. 11 or 17

19. exp "PREDICTIVE VALUE OF TESTS"/

20. (receiver operat$ adj2 curve).ab,ti.

21. (prognos$ or predict$ or course$).mp. [mp=title, original title, abstract, name of substance, mesh subject heading]

22. diagnostic test.mp.

23. exp MORTALITY/

24. exp ROC Curve/

25. exp "Sensitivity and Specificity"/

26. exp Follow-Up Studies/

27. 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26

28. 18 or 27

29. limit 28 to (human and english language and all adult <19 plus years> and yr=1980-2004)

Appendix 3 : QUADAS Tool

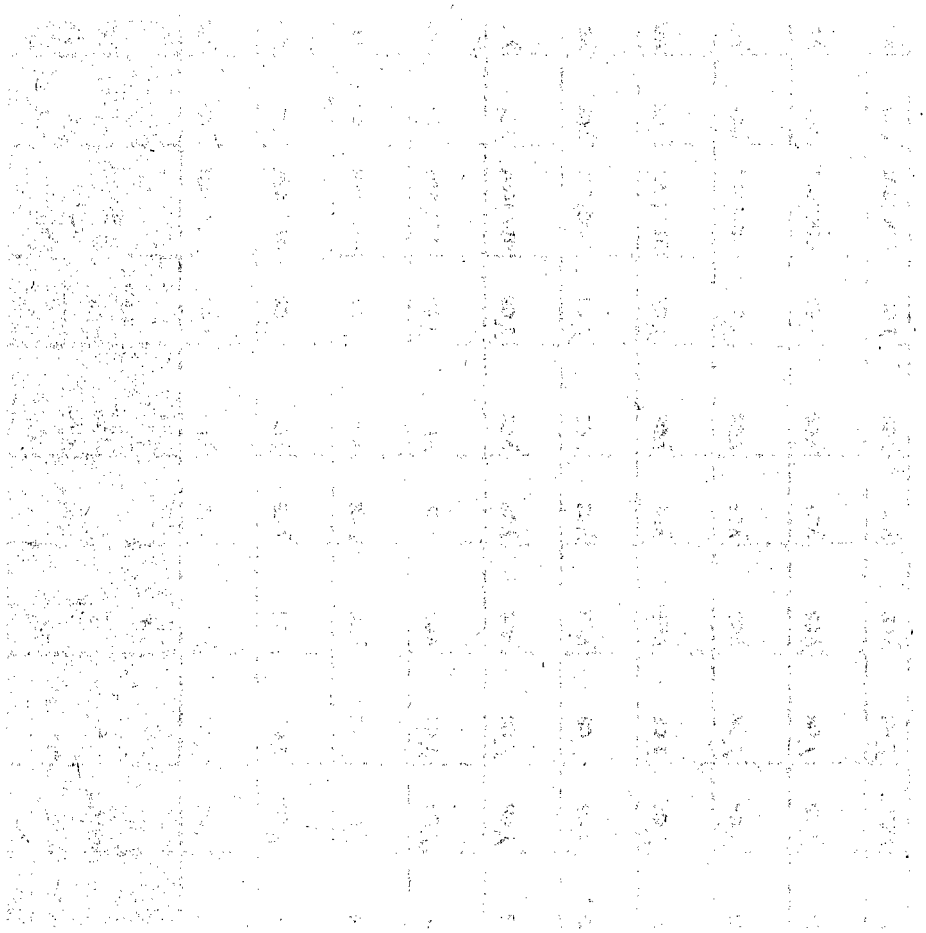
Item
1. Was the spectrum of patients representative of the patients who will receive the test in practice?
2. Were selection criteria clearly described?
3. Is the reference standard likely to classify the target condition correctly?
4. Is the time period between reference test and index test short enough to be reasonably sure that the target condition did not change between the two tests?
5. Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?
6. Did patients receive the same reference standard regardless of index test result?
7. Was the reference standard independent of the index test (i.e. the index test did not form part of the reference standard)?
8a. Was the execution of the index test described in sufficient detail to permit its replication?
8b. Was the execution of the reference standard described in sufficient detail to permit its replication
9a. Were the index test results interpreted without knowledge of the results of the reference standard?
10 Were the same clinical data available when test results were interpreted as would be available when the test is used in clinical practice?
11 Were uninterpretable /intermediate test results reported?
12. Were withdrawals from the study explained?

Additional questions were posed in the context of this review:

13a. Was the composition of the panels of serum markers reported in full?

13b. Was any formula derived for the panel of serum markers reported in full?

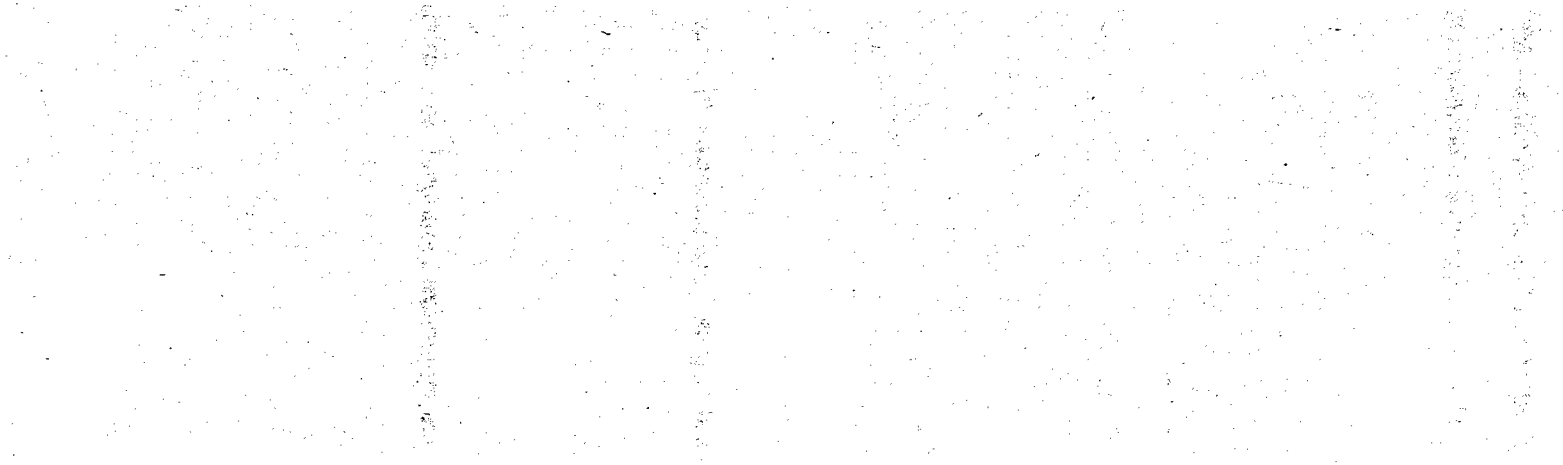
14. Was there validation in a separate cohort of patients of the panel of serum markers performance



Appendix 4 : Results of QUADAS tool in HCV SR

Author:	Rep sample	Select criter:	Ref Test Approp:	Ref/ index Test Time Short:	Verifi c	Verific With Same Ref Test:	Ref / Index Tests Indep:	Ref Test Reprod:	Index Test Blind	Ref Test Blind:	Data Same as in practice	Results Report:	Withdrawal explained:	Index tests: comp.**	formula Of Index Score	Valid Of score:
Imbert-Bismut	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref*	yes	yes	no	Yes
Poynard	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes - not all ref	yes	yes	no	No
Wai	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes - not all ref	no	yes	yes	yes
Leroy	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes - not all ref	yes	yes	yes	No
Sud	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	yes	yes	yes	Yes
Rossi	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	no	yes	no	Yes
Forns	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	yes	yes	no	Yes
El-Shorgaby	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	yes	yes	no	No
Thabut	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	no	yes	no	Yes
Calvez	Yes	yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all	no	yes	no	Yes

												ref				
Patel	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	yes	yes	no	Yes
Rosenberg	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	yes	yes	yes	Yes
Kaul	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	yes	yes	yes	Yes
Fortunato	Yes	Yes	Yes	yes	yes	yes	Yes	unclear	yes	yes	n/a	yes- not all ref	no	yes	yes	Yes



Appendix 5 : Search strategy for NAFLD SR

1. *Hyperlipidemia/ or *Hypertriglyceridemia/ or *Metabolic Syndrome X/ or *Obesity/ or *Insulin Resistance/ or *Diabetes Mellitus, Type 2/ or metabolic syndrome.mp. or *Hypertension/

2. fatty liver.mp. or exp Fatty Liver/

3. NAFLD.mp.

4. NASH.mp.

5. steatohepatitis.mp.

6. steatosis.mp.

7. non-alcoholic.mp.

8. 1 or 2 or 3 or 4 or 5 or 6 or 7

9. fibrosis.mp.

10. cirrhosis.mp.

11. $hepatitis.mp.

12. steatosis.mp. [mp=title, original title, abstract, name of substance word, subject heading word]

13. inflammation.mp.

14. 9 or 10 or 11 or 12 or 13

15. serum markers.mp. or exp Biological Markers/

16. diagnosis/ or "diagnostic techniques and procedures"/ or "laboratory techniques and procedures"/

17. $invasive.mp.

18. exp Liver Function Tests/

19. exp DIAGNOSTIC IMAGING/

20. predict$.mp.

21. marker$.mp.

22. surrogate.mp.

23. 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22

24. 8 and 14 and 23

25. limit 24 to (humans and english language)

26. limit 25 to "all adult (19 plus years)"

Appendix 6 : Results of QUADAS tool in NAFLD SR

Author	Q1 Rep sample	Q2 Select Criter	Q3 Ref Test Approp	Q4 Ref/index test time short	Q5 Verific	Q6 Verific with same Ref Test	Q7 Ref/Index tests Indep	Q8 Ref Test Reprod	Q9a Inde Test Blind	Q9b Ref Test Blind	Q10 Data same as in pract	Q11 Results report	Q12 Withdra-wal explained	Q13a Index tests comp	Q13b Index Score	Q14 Valid of score
Angulo	Yes	Yes	Yes	Yes	Yes	Yes	Yes	unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	no
Rosenberg	Yes	Yes	Yes	Yes	Yes	Yes	Yes	unclear	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Sakugawa	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	No	Yes	Yes	n/a	No
Albano	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	No
Mofrad	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	n/a	n/a
Shimada	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Dixon	No	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Beymer	No	Yes	Yes	Yes	Yes	Yes	Yes	unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Bugianesi	Yes	Yes	Yes	Yes	Yes	Yes	Yes	unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Dixon	No	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Hui	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	yes	n/a	n/a
Guidorizzi	Yes	Yes	Yes	Yes	Yes	yes	Yes	Unclear	Yes	Yes	Yes	Yes	No	Yes	n/a	n/a
Suzuki	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Angulo	No	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Marchesini	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Hashimoto	Yes	Yes	Yes	Yes	Yes	Yes	Yes	unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Ong	No	No	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Ledinghen	Yes	Yes	Yes	Yes	Yes	Yes	Yes	unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Ratziu	No	Yes	Yes	Yes	Yes	Yes	Yes	unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	no

Sorrentino	No	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Crespo	No	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Fierbinteanu-Braticevici	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Loguercio	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Santos	No	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Yesilova	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Koruk	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Hartleb	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a
Chitturi	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a	n/a
Brunt	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	n/a	n/a	n/a

Appendix 7 : SOP of ELF

Equipment used
Siemens Diagnostics Immuno 1 immunoanalyser
Sample cups
Pipette (200µl and 1000µl)
Sterile, filter pipettes and tips

Reagents (see MSDS for further safety details)
Deionised water
Aseptol (2% solution)

ELF assay reagents (HA, TIMP-1 and P3NP)

Cuvette wash 1
Cuvette wash 2
IMP wash
Reagent probe fluid
Sample probe fluid
Substrate
Substrate diluent
mIMP buffer
mIMP

Controls
HA low (50 ng/ml) stored at 4-8°C as a lyophilised powder
HA medium (200 ng/ml) stored at 4-8°C as a lyophilised powder
HA high (600 ng/ml) stored at 4-8°C as a lyophilised powder
TIMP-1 (low) 500 ng/ml) stored at 4-8°C as a lyophilised powder
TIMP-1 (high) 1000 ng/ml) stored at 4-8°C as a lyophilised powder
P3NP low (7.0 ng/ml) stored at 4-8°C as a lyophilised powder
P3NP high (70ng/ml) stored at 4-8°C as a lyophilised powder

Reagent preparation and use
The following reagents require preparation. Before carrying out any preparation, check the amounts loaded on the Immuno 1 to see if further preparations are required.
NOTE: for the substrate, 3x the sample number will be required to carry out the full panel of markers.

Generic reagents
Substrate
Add the entire contents of the substrate diluent to the substrate reagent bottle, mix gently (invert a couple of times) and leave at room temperature for 5 minutes to ensure full solubilisation (with occasional mixing).
Pour the entire contents into a new substrate tower (through the top hole, replacing the bung). Then remove the lower bung and load onto the Immuno 1.
Go to the "REAGENT" page and enter "N" in the relevant position (under "PDT") and press "ENTER" to load the new reagent which will appear as "175" reactions left.
Ensure the substrate is kept either on the Immuno 1 or in a fridge and in the dark once solubilised. Prepared substrate can be used for up to 1 week.

ELF specific reagents

HA reagents 1 and 2
Remove from the fridge HA reagents 1 and 2 (brown, glass bottles) sufficient for the number of samples to be analysed.
Leave at room temperature for 15 min
Reconstitute each in 8ml deionised water
Leave for 30 min on bench (mix occasionally)
Pipette into reagent tower (ensure the reagent goes in the correct compartment)
Put in fridge to chill (10 min)
Ensure prepared HA reagents are stored either on the Immuno 1 or in a fridge
Prepared reagent can be used for up to 3 days
Go to loading fibrosis reagents (see below)

Quality controls
Remove from the fridge QC samples:
HA Low (50) / Medium (200) / High (600)
TIMP-1 Low (500) / High (1000)
P3NP Low (7) / High (70)
Reconstitute each in 2 ml deionised water
Leave on bench for 15 min (mix occasionally)
Keep all QC samples in the fridge when prepared (for up to 7 days)

Other reagents
All the remaining reagents are ready to use and require no preparation. Ensure they are all stored at the correct temperatures as indicated on the labels.

Instructions/ Method
'Only those personnel (or appropriately supervised trainees) trained (or being trained) in the use of relevant reagents and equipment should carry out this procedure.'

Reagent and waste check (before every run)
Wash buffers
Check levels of all Wash buffers (Cuvette wash 1, Cuvette wash 2 and IMP wash) - base of the Immuno 1
Ensure all are full (above the label, below the cap) or have sufficient to carry out the work planned

Waste check
Take out the waste pot and empty into the sink with running, cold water to wash it away
Empty the small waste container
Refit both waste containers ensuring all three tubes/wires are reconnected to the large waste pot

Substrate check
Ensure there is sufficient substrate for the work to be carried out (go to Reagent Inventory page)
Remember multiple tests may be carried out on individual samples (e.g. HA, TIMP-1 and P3NP)

Probe fluids
Check levels of Sample Probe and Reagent Probe Fluids – top of the Immuno 1.
Ensure both are full
Fill each up to the shoulder of the bottle, try to do this without lifting the probe out of the fluid (if this does happen use "PRIME" in the "MAINTENANCE" menu to pump through the air bubbles)

mIMP and buffer
Check the levels of the mIMP and buffer (if already loaded)
If not loaded, load mIMP and buffer onto machine (from fridge). Check there is sufficient for work to be carried out
Ensure the magnetic beads are suspended (by swirling gently with the cap on)

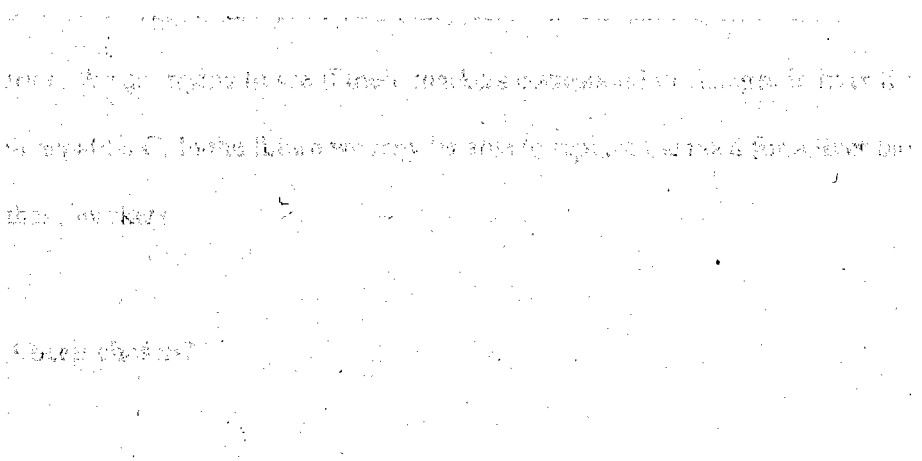
Loading ELF reagent towers (before every run)
Each tower has enough volume for 100 tests. If required, load new towers into the reagent carousel as follows:
HA reagents 1 and 2 made up earlier and the other marker reagents from the fridge in the same order as the Reagent inventory page (ensure they are only put into these positions)
Go to the Reagent inventory page and update any changes by entering "100" in the number column manually as the reagents are not recognised by the machine (no bar codes).

Wash jets check and prime (before the first run of the day)
Move wash jets to the "out" position and remove reaction tray (inspect the tray for residual substrate etc. and replaced if required) and place to one side
Replace with the reaction tray marked as "FDT ONLY" leave the cover off the reaction tray so the fluid delivery can be observed
Put wash jets back into position and clean all wash jets by removing them and clean the inside (using a syringe) and make sure the outside is wiped clean of dried buffer
Ensure no tissue is left on the jets
Go to "MAIN MENU" and select "MAINTENANCE" then "PRIME"
Enter 50x each probe fluid and 15x each wash jet (maximum)
Select "PRIME ALL"
Whilst priming, watch all the jets to ensure they are all injecting buffer and drawing it back up again
If any jet looks like this may not be happening, then it may be necessary to repeat cleaning as above
If required (e.g. if it doesn't look like the jets are injecting buffer properly) then repeat the prime on the suspect jets as many times as required
If the vacuum does not appear to be working (reaction cuvettes are overflowing / not emptying properly) check the vacuum gauges and the silicone tubing in the wash stations behind the drop down panel
Replace any silicone tubing which appears damaged or dirty
Check the syringes aren't taking in air bubbles, if so they may need replacing (see maintenance book)

Fluid delivery test, FDT (before the first run of the day)
Check there is sufficient FDT reagent in the tower (carry out a visual check - position 22 on the carousel). If not, fill with IMP wash buffer
Put tap water into a sample cup and place in rack 1, position 1 and load onto machine in position 1 (black base)
Go to "MAIN MENU" then "ORDER MENU" and select "ORDER ENTRY" and press "ENTER"

Ensure the load list number is not "00" if it is, then change the load list number to "01", "02", "03" etc. Press "ENTER"
Enter "100" 20 times on the order entry
This will tell the machine to do 20 FDT tests
"ENTER" then select "RUN" and "ENTER" the relevant load list to start the FDT
A grating noise will signal the raising of the sample probe
Select the "SAMPLE" screen and note the time of the first sample
During the FDT the levels of each of the vacuums should be verified along with the correct running temperature of the refrigeration unit
Visually ensure all the wash jets are functioning correctly
After 6 minutes, stop the run (SHIFT and STOP) just after the sample probe has delivered the sample and come back out of the reaction tray
Wait for the moving bits to stop (when the sample probe is back in the starting position) and move the wash jets out of the way and remove the "FDT" reaction tray
Using the guide card examine the tray and see if each component of the fluid delivery is working ok
If there is any failure (usually a wash buffer) it is usually due to
jets being blocked (clean and "PRIME" again)
vacuum failure (contact service engineer)
tubes requiring replacement (replace as outlined later)
repeat all the above FDT with second "FDT tray"
If successful, remove the FDT reaction tray and wash out for use again
Replace the clean reaction tray, replace the cover and put the wash jets back into position

The above procedures should ensure that air bubbles are removed from all tubing, the tubing is in good order and the jets are dispensing and withdrawing the correct amounts of sample, buffers etc. and the reagents are being held at the correct temperature.



Appendix 8 : Information sheet given to participants in clinical study

LREC No. 04/Q1701/58

Serum and urine markers of liver fibrosis

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

To assess the degree of inflammation and scarring (fibrosis) in the liver one of the tests we use is a liver biopsy. This test allows us to examine a small piece of liver tissue under a microscope. Currently it is the best test available but we are interested in finding other ways of measuring inflammation and scarring in the liver. This study involves examining chemicals (markers) in your blood and urine. We are trying to see if these markers correspond to changes in liver tissue of patients with hepatitis C. In the future we may be able to replace the need for a liver biopsy by measuring these markers.

Why have I been chosen?

Our study involves patients with chronic hepatitis C who require a liver biopsy as part of their standard management plan.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part and what do I have to do?

If you agree to take part in the study we will contact you within the next four months. We will invite you to see us in a clinic at the Wellcome Trust Clinical Research Facility where we will ask some simple questions, examine you, perform blood and urine tests.
In order to get meaningful results from the urine test we will ask you to avoid the following from the time indicated, until the end of your clinic visit.

From one week before avoid	Medicines (including herbal preparations), cannabis containing substances and dietary supplements
From the day before avoid	Fish, seafood, spicy food and alcohol
From the day of consultation avoid	Tea, Coffee and very fatty or fried foods, for example a cooked breakfast

You may eat on the day of the study (following the restrictions listed above), but we request that you drink only water on the day of the study, both prior to attending the clinic and during your visit to the clinic.

What are the possible disadvantages and risks of taking part?

There are no risks of taking part in the study. The only disadvantages are giving a blood test and making some minor changes to your lifestyle (as listed above) from a week before the study day until the end of your clinic visit.

What are the possible benefits of taking part?

There will be no immediate benefit to your care if you participate in the study. This study will help the management of patients with hepatitis C and other causes of liver disease in the future. If we can find effective markers for inflammation and scarring in the liver we may be able to reduce the number of people having a liver biopsy. Additionally, we may have more accurate tests for assessing liver damage before, during and after treatment.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. Your GP will be informed of your participation in the study.

What will happen to the results of the research study?

We aim to publish the study in a peer reviewed medical journal. You will be able to gain a copy of the publication. You will not be identified or be identifiable in any future publication.

Who is organising and funding the research?

The study is funded by a pharmaceutical company called Pfizer and has been organised by Dr. William Rosenberg at Southampton General Hospital. The samples we collect will be stored at Pfizer for five years. If you wish to withdraw from the project at any stage you simply need to contact Dr. Guha or Dr. Rosenberg (numbers listed below). In the event of withdrawal, we will be able to locate your samples and have them destroyed to prevent further use.

Contact for Further Information

Further information can be obtained by contacting Dr. Neil Guha at the Wellcome Clinical Research Facility at Southampton General Hospital.

Thank you for your time.

Dr Neil Guha Tel 023 8079 4989

Dr William Rosenberg Tel 023 8079 6883

Appendix 9 : Methods for NMR spectroscopy on animal tissue

Sample preparation

For Magic angle spinning (MAS) NMR experiments 20 mg of tissue was taken and packed into 4 mm outer diameter zirconia rotors with a spherical insert and a Kel-F cap after soaking in D_2O. For the water soluble experiments extraction was achieved with 1 ml of solution containing 50 % acetonitrile and 50 % water. This procedure was repeated three times followed by centrifugation of samples at 10,000 rpm for 6 mins. The supernatant was decanted and acetonitrile was removed under N_2 gas before freeze drying the samples. In the lipid extraction experiments samples were treated with 1 ml of solution containing 75 % $CHCl_3$ and 25 % methanol. Three repeat washes were carried out and centrifugation was performed at 10,0000 rpm for 10 mins. The solvent was removed by drying under a stream of N_2 gas.

Prior to NMR analysis, lipid soluble extracts were reconstituted in 600 µl of solvent containing 70% $CDCl_3$ and 30% CD_3OD. Water soluble extracts were reconstituted in D_2O containing 0.05 % TSP as chemical shift reference.

NMR spectroscopy

All NMR experiments were carried out on a Bruker DRX-600 spectrometer (Bruker Biospin, Germany) operating at a ^1H frequency of 600.13 MHz.

MAS-NMR spectroscopy was performed at 283K and samples were spun at 5 kHz at an angle of 54.7°. The following CPMG sequence was used to suppress signals from macromolecules and other short T_2 components: relaxation delay [RD-90°-(τ-180°-τ)$_n$-ACQ]. A spin-spin relaxation delay of 2nτ of 200ms was used and water suppression was applied during a 2s relaxation delay. Spectra acquired were the sum of 256 transients with 32,000 data points and a spectral width of 12,000Hz.

For the water soluble extracts a standard one dimensional pulse sequence was applied at 300K: [RD-90°-t_1-90°-t_m-90°-ACQ] where t_m was set to 100ms and t_1 was set to 3μs. Water suppression was achieved through irradiation of the water peak during a relaxation delay of 2s. Spectra acquired were the sum of 256 transients collected with 32,000 data points and a spectral width of 12,000Hz.

For the lipid extraction a standard pulse sequence was applied with the temperature regulated at 300K. Water suppression was achieved through pre-saturation during a relaxation delay of 3.3s. Spectra acquired were the sum of 128 transients with 32,000 data points and a spectral width of 12,000Hz.

NMR data processing

All spectra were manually corrected for phase and baseline distortions. The MAS-NMR spectra were referenced to the glucose H1 proton at 5.23ppm. The water extract spectra were referenced using TSP whilst the lipid extract spectra were referenced to the $CDCl_3$ resonance at 7.26ppm. All spectra through the range 0.2-10.0ppm (excluding the water region from 4.5-6.0ppm) were reduced to 245 regions each 0.04ppm wide using AMIX software (Bruker Analytik, Germany) and the signal intensity for each region was integrated.

Appendix 10 : Publications arising from work in this thesis

1. Parkes J, Guha IN, Roderick P, Rosenberg W. Performance of serum marker panels for liver fibrosis in chronic hepatitis C. *J.Hepatol.* 2006;**44**:462-74.

2. Guha IN, Parkes J, Roderick PR, Harris S, Rosenberg WM. Non-invasive markers associated with liver fibrosis in non-alcoholic fatty liver disease. *Gut* 2006;**55**:1650-60.

3. Guha IN and Iredale JP. Clinical aspects of cirrhosis. *Textbook of hepatology from basic science to clinical practice*, 2007.

4. Iredale JP and Guha IN. Evolution of fibrosis to cirrhosis. *Textbook of hepatology from basic science to clinical practice*, 2007.

5. Guha IN, Parkes J, Roderick PR, et al. Non-invasive markers of fibrosis in non-alcoholic fatty liver disease: validating the European Liver Fibrosis Panel and exploring simple markers. *Hepatology* 2008; *In press*.