

**IMAGING TECHNIQUES IN
CHRONIC LIVER DISEASE:
APPLICATIONS OF PROTON MAGNETIC
RESONANCE SPECTROSCOPY**

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THESIS ABSTRACT

Proton magnetic resonance spectroscopy (^1H MRS) was used to assess chronic liver disease non-invasively. ^1H MRS was optimised to study intact liver tissue *in vitro* and used to derive hepatic metabolic profiles in murine models of non-alcoholic fatty liver disease. The technique was applied to liver biopsy specimens from 47 patients with chronic hepatitis C. *In vitro* MRS data were used to inform analysis and aid interpretation of *in vivo* MR spectra. Findings were validated in a separate cohort of 59 patients *in vivo*. Diagnostic performance of other emerging non-invasive techniques, ultrasound and serum markers, were compared with histology in 67 patients with chronic hepatitis C. Finally, 38 patients underwent comparative ^1H MRS, transient elastography and AST to platelet ratio index (APRI).

Multivariate pattern recognition enabled distinction of murine liver tissue by strain and diet. There was correct prediction of histologically-defined steatohepatitis from steatotic and normal samples.

Disease severity was correctly predicted in 46 of 47 human liver samples. Lipid and choline resonances contributed predominantly to statistical models. ^1H MRS indices of total lipid, lipid saturation and polyunsaturation, and choline were derived *in vitro* and validated *in vivo*. Total lipid assessed by ^1H MRS was higher in patients with genotype 3 than genotype 1. The degree of fatty acid polyunsaturation decreased with fibrosis stage and steatosis.

In 67 patients with hepatitis C, APRI combined with either transient elastography or the serum panel marker, ELF, effectively predicted fibrosis stage, while three or more tests combined led to redundancy of information. Microbubble ultrasound performed least well. In the smaller cohort, ^1H MRS improved prediction of hepatic fibrosis when combined with transient elastography or APRI.

In conclusion, assessment of hepatic lipid composition by ^1H MRS is of biological importance in chronic liver disease and complements data conveyed by other modalities.

To Rachael,
with love.

DECLARATION

I declare that the work presented in this thesis is my own.

Jeremy F. L. Cobbold

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LIST OF ABBREVIATIONS

¹H	proton
³¹P	phosphorus-31
ADC	apparent diffusion coefficient
ALT	alanine aminotransferase
APRI	AST to platelet ratio index
AST	aspartate aminotransferase
AUROC	area under the receiver operator characteristic
CHC	chronic hepatitis C
ChREBP	carbohydrate responsive element binding protein
CLD	chronic liver disease
CLR	choline to lipid ratio
CT	computed tomography
DDR	discoidin domain receptor
DWI	diffusion weighted imaging
ECM	extracellular matrix
ET	endothelin
FA	fatty acid
FGF	fibroblast growth factor
FID	free induction decay
GPC	glycerophosphocholine
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDL	high density lipoprotein
HNE	hydroxy 2,3 nonenal
HSC	hepatic stellate cell
HVPG	hepatic vein pressure gradient
IGF	insulin-like growth factor
LDL	low density lipoprotein
MAS	magic angle spinning
MDA	malondialdehyde
MMP	matrix metalloproteinase
MRE	magnetic resonance elastography
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid

MRS	magnetic resonance spectroscopy
MTP	microsomal triglyceride transfer protein
MUFA	monounsaturated fatty acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NMR	nuclear magnetic resonance
NO	nitric oxide
PC	phosphocholine
PDE	phosphodiester
PDGF	platelet-derived growth factor
PME	phosphomonoester
PPAR	peroxisome proliferator-activated receptor
PtC	phosphatidylcholine
PUFA	polyunsaturated fatty acid
PUI	polyunsaturation index
RF	radiofrequency
ROC	receiver operator characteristic
ROS	reactive oxygen species
SI	saturation index
SNR	signal to noise ratio
SREBP	steroid responsive element binding protein
T₁	longitudinal (“spin-lattice”) relaxation time
T₂	transverse (“spin-spin”) relaxation time
tCho	total choline-related compounds
TE	transient elastography
TGF	tissue growth factor
TIMP	tissue inhibitor of metalloproteinase
TKR	tyrosine kinase receptor
tPA	tissue plasminogen activator
TR	repetition time
UI	unsaturation index
US	ultrasound
VLDL	very low density lipoprotein

1 INTRODUCTION

1.1 The clinical problem

Chronic liver disease is a major cause of morbidity and mortality worldwide. The presence of chronic inflammation and consequent fibrosis leads to the development of cirrhosis and its complications. End-stage liver disease accounts for one in 40 deaths worldwide, according to World Health Organisation estimates from 2002 (Perz *et al.*, 2006). In the United States, cirrhosis accounted for 25 000 deaths in 1998 and the worldwide prevalence of cirrhosis has been estimated at up to 1% (Schuppan and Afdhal, 2008).

Chronic liver disease has a varied aetiology, including viruses, such as hepatitis B (HBV) and hepatitis C (HCV). Worldwide, over half a billion people may be chronically infected with either of these viruses (World Health Organisation, 2000; World Health Organisation, 2008). Metabolic causes include the increasingly prevalent non-alcoholic fatty liver disease (NAFLD). Toxic causes, such as excess alcohol consumption, and autoimmune disorders, such as primary biliary cirrhosis and autoimmune hepatitis, contribute to the disease burden. Over half of the deaths attributable to cirrhosis and nearly 80% of those attributable to primary liver cancers occur in those who have chronic HBV and HCV infection (Perz *et al.*, 2006), while in many developed nations excess alcohol consumption is the commonest cause.

Chronic liver disease takes many years to progress from inflammation, associated with hepatocellular injury, to fibrosis and requires long-term exposure to the causative agent. Progressive scarring or fibrosis develops during the period of time between initiation and end-stage disease. The resulting pre-cirrhotic fibrosis is a target for therapies aimed at reducing the rate of progression to cirrhosis, or even reversal of fibrosis (Friedman, 2003).

Effective antiviral therapies and the advent of antifibrotic drugs have led to increasing demand for non-invasive, accurate and reliable biomarkers of hepatic disease severity. It is recognised

that the current “gold standard”, histological analysis of liver biopsy, has limitations and engenders risk to the patient. Sampling variability and the subjective interpretation of scoring systems means that consistency and the representation of the true disease state is questionable. The procedure frequently causes discomfort and there is a risk of morbidity and a small, but significant, mortality rate (Gilmore *et al.*, 1995). These risks preclude frequent serial biopsies in most cases. Emerging non-invasive imaging-based technologies, particularly using ultrasound and nuclear magnetic resonance (NMR), allow a quantitative assessment of different aspects of disease from metabolic markers of inflammation, through assessment of fibrotic load, markers of portal hypertension and prognostic indicators of the complications of cirrhosis (Cobbold *et al.*, 2006).

There is a clear need for safe and effective non-invasive markers of hepatic fibrosis, both for the management of individual patients and for the development of new antifibrotic therapies. Imaging modalities have the potential to fulfil this role.

1.2 Chronic liver disease

Chronic liver disease occurs over months and years. Resultant morbidity and mortality are largely associated with the development of end-stage liver disease. Cirrhosis is defined as the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury, leading to portal hypertension and end-stage liver disease (Schuppan and Afdhal, 2008). The condition is indolent and frequently asymptomatic until the development of complications, occurring late in the disease process. Such complications include variceal haemorrhage, ascites, spontaneous bacterial peritonitis and hepatic encephalopathy. Cirrhosis is a risk factor for the development of hepatocellular carcinoma (Fattovich *et al.*, 2004).

1.2.1 The natural history of chronic liver disease

Chronic liver injury may lead to fibrosis as a result of accumulation of “scar” tissue. Liver injury leads to initiation and perpetuation of inflammatory processes, which, by a cascade of inter-related processes and pathways, leads to deposition of fibrous proteins, such as collagens (**Figure 1-1**). By convention, fibrosis has been considered potentially reversible, while the end-stage, cirrhosis, has been considered irreversible. However, with elimination of the cause of liver injury, a number of studies have demonstrated regression of all stages of fibrosis in animal models and in humans (Hammel *et al.*, 2001; Iredale *et al.*, 1998; Iredale, 2001; Kweon *et al.*, 2001; Poynard *et al.*, 2002). Elucidation of the process of fibrogenesis enables markers of disease severity and potential targets for therapeutic intervention to be developed.

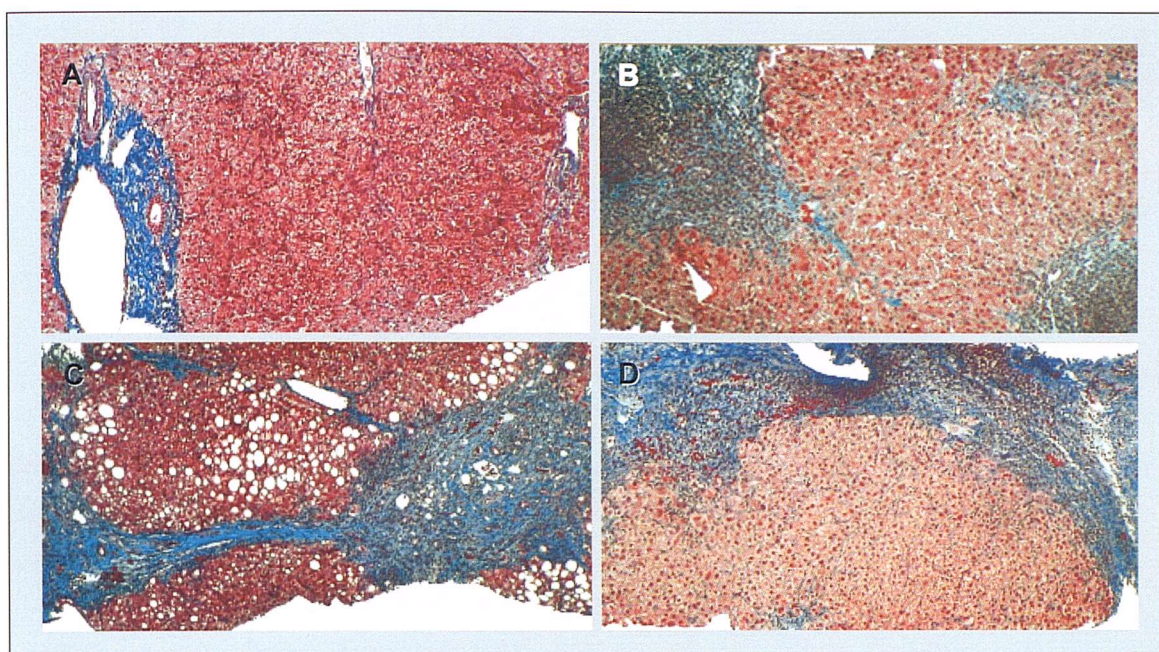


Figure 1-1. Histology of normal liver, fibrosis and cirrhosis

Representative histological images (magnification x200, Masson's trichrome staining) of A, normal liver; B, mild to moderate fibrosis (METAVIR F=2, Ishak stage 3); C, moderate "bridging" fibrosis (METAVIR F=3, Ishak stage 4); D, cirrhosis (METAVIR F=4, Ishak stage 5 or 6). Adapted from www.PHEONIXCME.org

1.2.2 Normal liver

Normal liver consists of rows of hepatocytes surrounded by fenestrated endothelium within sinusoids containing venous blood. Kupffer cells (tissue macrophages) are present within sinusoids. The space of Disse lies between the hepatocytes and the endothelial layer and contains a basement membrane-like matrix (extracellular matrix- ECM), which is essential for maintenance of functioning liver cells. Hepatic stellate cells (HSC), also called Ito cells or perisinusoidal cells, are present in the space of Disse and are thought to be the principal cell type involved in hepatic fibrogenesis (**Figure 1-2**) (Friedman, 2003).

1.2.3 Anatomy of liver fibrosis

In chronic liver disease, the composition of the ECM is altered with increasing fibrosis to contain higher quantities of collagens, and a change in conformation from low-density basement membrane-like lattice matrix, through which solutes and growth factors are freely diffusible, to

a disordered interstitial type matrix, which interferes with hepatic function (**Figure 1-2**) (Friedman, 2003).

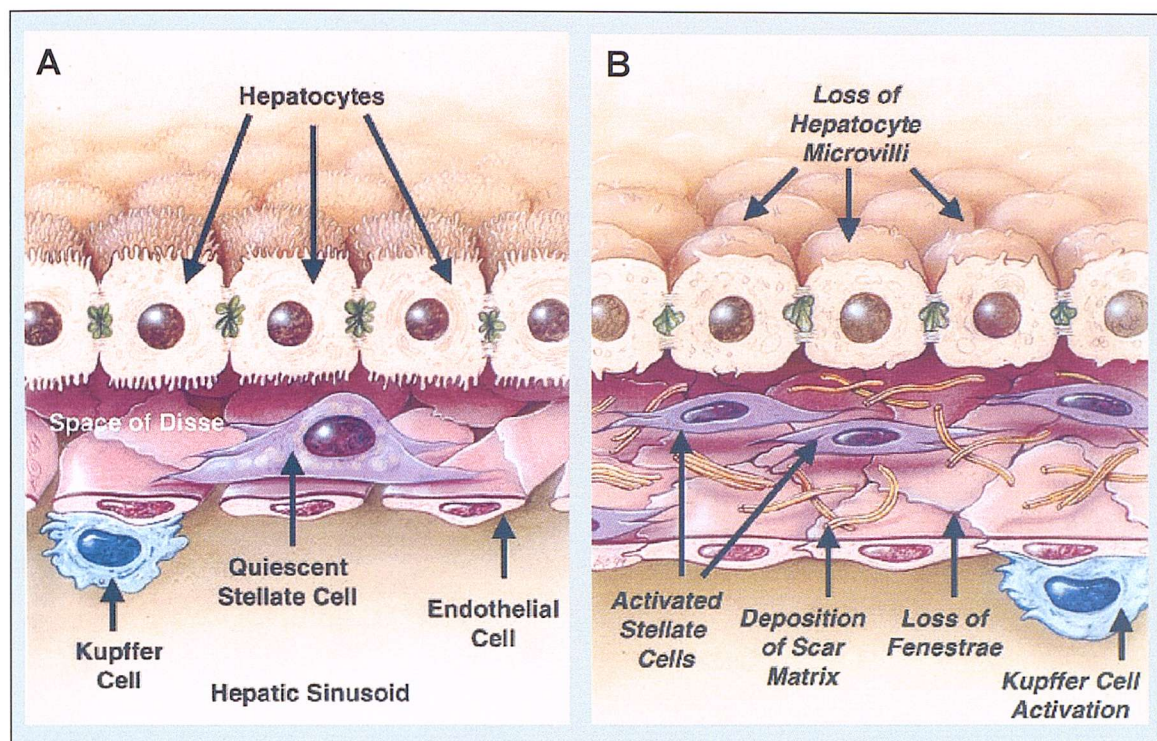


Figure 1-2. Schematic of sinusoidal structure in health and fibrotic liver disease.

Panel A shows the sinusoidal space in healthy liver, while panel B shows some of the changes that occur with the development of fibrosis, resulting in deterioration of hepatic function. Adapted from Friedman 2000.

1.2.4 Mechanism of fibrogenesis (Friedman, 2003; Friedman, 2008)

The HSC has a central role in the pathogenesis of liver fibrosis. These cells comprise 15% of liver cells and are thought to be derived from neural crest tissue, on account of the expression of neural crest markers, including glial fibrillary acidic protein and nestin (Niki *et al.*, 1999).

HSCs are activated following liver injury from a relatively quiescent lipid and vitamin A-storing phenotype to a myofibroblastic phenotype capable of proliferation, contraction and fibrogenesis. Recently, other myofibroblastic cell populations have also been shown to be involved in

fibrogenesis, including portal fibroblasts (Beaussier *et al.*, 2007) and bone marrow-derived cells (Forbes *et al.*, 2004).

HSC activation occurs through two stages; initiation and perpetuation, each of which involves characteristic pathways, as described by Friedman.

1.2.4.1 *Initiation*

Initiating events may consist of any chronic perturbation of hepatic homeostasis, which is often, but not always, associated with the presence of inflammatory cells. Such perturbations of hepatic homeostasis may lead to the net over-production of unstable reactive oxygen species (ROS) derived from hepatocytes, macrophages, stellate cells and inflammatory cells. ROS are potent mediators of the initiation and perpetuation of liver injury, and cause lipid peroxidation of cellular membranes. The importance of lipid peroxidation extends beyond the activation of stellate cells and is also of considerable importance to the development of insulin resistance and hepatic steatosis, as described in more detail in **section 1.5.3**.

Cell death is both a cause and a consequence of altered hepatic homeostasis that leads to the upregulation of the inflammatory response and activation of fibrogenic pathways. Cell death occurs in two modes, apoptosis and necrosis, with differing mechanisms and local responses. Apoptosis, or programmed cell death, of parenchymal cells represents an important inflammatory stimulus, which can activate HSCs (Jaeschke, 2002). Necrosis, which is often found to coexist with apoptosis in histological sections, is also a potent activator of inflammation and fibrogenesis, associated with redox imbalance (Poli, 2000).

During initiation (or the pre-inflammatory phase), there is immediate early gene expression leading to changes in the phenotype of HSCs, which results in increased responsiveness to cytokines and other local stimuli. Injury to cells nearby causes changes in cellular homeostasis and to ECM composition. Release of reactive oxygen intermediates from damaged hepatocytes stimulates local HSCs and the activity of HSCs in culture has been shown to increase upon

depletion of antioxidants. In addition, culture medium taken from hepatocytes undergoing oxidative stress results in increased proliferation and collagen synthesis, when applied to cultured HSCs (Svegliati *et al.*, 1998). Damage to endothelial cells also contributes to HSC activation by the production of a splice variant of cellular fibronectin (Jarnagin *et al.*, 1994) and by converting transforming growth factor β 1 (TGF β) to its active form through activation of plasmin (Friedman, 1999). Furthermore, the infiltration and activation of Kupffer cells (KCs) is temporally associated with expression of HSC activation markers. TGF β and other cytokines from KCs stimulate the synthesis of ECM and KCs also release ROS in response to injury.

1.2.4.2 *Perpetuation*

Perpetuation of HSC activation is comprised of a number of cellular responses, which lead to increase expression of, and responsiveness to, growth factors. Perpetuation is a dynamic process, occurring along a number of pathways associated with different HSC responses. These responses include release of largely proinflammatory cytokines, proliferation and increased contractility of HSCs, chemotaxis of inflammatory cells and the net deposition of pathological ECM.

1.2.4.2.1 *Cytokine release*

A large number of autocrine and paracrine cytokines are released during activation, including TGF β , platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), platelet activating factor and endothelin-1 (ET-1) (Pinzani *et al.*, 1994; Pinzani and Marra, 2001). Neutrophil and monocyte chemoattractants are also released leading to increased local inflammatory infiltration (Marra *et al.*, 1998).

1.2.4.2.2 *Proliferation*

The expression of a number of mitogenic factors and upregulation of their tyrosine kinase receptors (TKRs) leads to proliferation of HSCs. PDGF is a potent stimulator of proliferation that, together with its receptor, has been shown to be up-regulated following liver injury (Pinzani *et al.*, 1994). A TKR, discoidin domain receptor-2 (DDR-2) binds to fibrillar collagen as its ligand, provoking a biochemical cascade, leading to activation of matrix

metalloproteinase-2 (MMP-2), which is itself a HSC mitogen (Olaso *et al.*, 2001; Olaso *et al.*, 2002).

1.2.4.2.3 *Contractility*

Activation of HSCs also leads to increased contractility of the myofibroblastic phenotype and impendence of portal blood flow by constriction of sinusoids and overall contraction of the cirrhotic liver. This is mediated by endothelin-1 (ET-1) (Racine-Samson *et al.*, 1997), the action of which is opposed by nitric oxide (NO), also produced by a number of liver cell types. Progressive development of intrahepatic shunts occurring in concert with the constriction of hepatic sinusoids has been postulated to require angiogenic responses.

1.2.4.2.4 *Chemotaxis*

Activated HSCs migrate to areas of hepatic injury and a number of growth factors have been identified within the liver that have chemoattractant as well as mitogenic properties. These include PDGF, IGF-1, ET-1, monocyte chemoattractant protein 1 and the chemokine receptor CXCR3 (Bonacchi *et al.*, 2001; Gentilini *et al.*, 2000; Marra *et al.*, 1998; Tangkijvanich *et al.*, 2001).

1.2.4.2.5 *Matrix accumulation and degradation*

HSCs, platelets and KCs secrete TGF β 1, which is activated by MMPs and tissue plasminogen activator (tPA). TGF β receptors are upregulated upon activation of HSCs leading to increased responsiveness and increased manufacture of ECM components (Gressner *et al.*, 2002). The build up of pathological ECM or scar tissue is a dynamic process with competition between accumulation and degradation. Degradation may be divided into that of the normal, low density ECM, termed “pathological matrix degradation”, which is associated with worsening liver disease and degradation of the higher density fibrotic ECM, termed “restorative matrix degradation” which is associated with improvement in fibrosis and restoration of normal hepatic architecture (Friedman, 2003).

Matrix-metalloproteinases (MMP) are calcium dependent enzymes, which degrade collagens and other substrates. MMPs may be divided into categories dependent on their specific substrate, for example: collagenases, stromelysins, gelatinases and membrane-type MMPs (Poli, 2000). Activated HSCs express latent proenzymes of MMPs, which are then activated by proteolytic cleavage by urokinase or tissue plasminogen activator (He *et al.*, 1989). The hepatic tissue expresses tissue inhibitors of metalloproteinases (TIMP) specific to each MMP type, which inhibit MMP activity and help to localize MMP activity where it is required (Benyon and Arthur, 2001). Protein structures determine the binding of MMPs and their respective TIMPs according to strict ratios and determine the resultant activity. Accordingly, net quantities of these enzymes determine local collagenase activity.

In the progression from fibrotic to cirrhotic liver, there appears to be a relative deficiency in collagenase activity, such that degradation of type 1 collagen, the main component of pathological ECM is impaired. Marked increases in TIMP-1 and TIMP-2 have been observed in progressive fibrosis leading to decreased MMP activity. HSCs are thought to be the major source of these inhibitors, representing another role for HSCs in the pathogenesis of fibrosis (Iredale, 2001).

1.2.4.3 *Extracellular matrix*

The interaction between HSCs and the ECM also appears to be important in the initiation and perpetuation of fibrogenic processes by a number of positive feedback pathways. These include changes in membrane receptors, such as integrins, which promote migration and contraction, activation of cellular MMPs, and increases in matrix stiffness. It appears that a number of hepatic cell types are mechanosensitive, leading to stellate cell activation and upregulation of profibrogenic receptors, associated with increased matrix stiffness (Wells, 2008).

1.2.4.4 *Resolution of fibrosis*

For resolution of fibrosis to occur, removal of the injurious agent is prerequisite. Activation of HSCs has been shown to be crucial for the development of fibrosis, while in recovery and

resolution of fibrosis, the number of activated HSCs is reduced. The latter may occur as a result of reversion to the quiescent form or by apoptosis. Return to the quiescent form has been observed *in vitro* (Sohara *et al.*, 2002), but not *in vivo*. Apoptosis has been postulated as the predominant mechanism for removal of HSC activity. It has been shown that spontaneous apoptosis of HSCs occurs *in vitro*. Conversely, it has also been shown that MMP-2 levels correlate with apoptosis and may be stimulated by apoptosis (Preaux *et al.*, 2002) and that TIMP-1, by inhibition of MMP, leads to inhibition of activated HSC apoptosis (Murphy *et al.*, 2002).

1.3 Hepatic steatosis

Liver fibrosis is the key pathological feature of progressive liver disease. However, the accumulation of excessive hepatic triglyceride, hepatic steatosis, is increasingly recognised as an important factor in the pathogenesis of a number of chronic liver diseases, not simply an “innocent bystander”.

1.3.1 Definitions

Hepatic steatosis is a pathological lesion, defined as the presence of large and small vesicles of fat, predominantly triglycerides, accumulating within hepatocytes (Clark *et al.*, 2002). Hepatic steatosis is frequently associated with obesity, insulin resistance and dyslipidaemia in those who do not drink excessive alcohol, when it is termed non-alcoholic fatty liver disease (NAFLD) (Angulo, 2002). Hepatic steatosis may also be a result of secondary causes, which include alcoholism, hepatitis C virus, severe weight loss, total parenteral nutrition and drugs (such as amiodarone, diltiazem, tamoxifen, steroids and highly active antiretroviral therapy) (Sanyal, 2002). NAFLD may thus be considered a syndrome of various aetiologies, excluding alcohol and, by convention, hepatitis C.

1.3.2 Epidemiology

As there is no simple non-invasive test for NAFLD, the prevalence of NAFLD has been estimated by extrapolation from other studies. Angulo estimated the prevalence of NAFLD in the U.S. population to be around 30 million (or 21% of adults) in 2000 on the basis of the prevalence of obesity in the US population, multiplied by the proportion of those found to have hepatic steatosis in studies of obese people (Angulo, 2002). Clark and colleagues estimated the prevalence to be 31 million (or 23% of adults) in the U.S on the basis that 79% of patients with mildly abnormal liver function tests and no other cause described are likely to have NAFLD (Clark *et al.*, 2002).

1.3.3 Pathophysiology

The development of hepatic steatosis occurs when the rate of synthesis or import of fatty acids by hepatocytes exceeds the rate of export or catabolism. Such an imbalance can occur in a number of ways:

1.3.3.1 *Increased uptake of fatty acids by hepatocytes*

Hepatic steatosis is strongly associated with obesity. Adipose tissue is a metabolically active tissue and is a source of a number of mediators of carbohydrate and lipid metabolism, including leptin, adiponectin, apolipoprotein E, and lipoprotein lipase. It is also a source of a number of cytokines, including TNF α , IL-6 and TGF β (Brunt, 2004). TNF α levels rise in obesity, and TNF α activates I κ B kinase β , which inhibits phosphorylation of insulin receptor substrates, one of the mechanisms leading to insulin resistance. This reduces the insulin-mediated suppression of hormone sensitive lipase, increasing the release of fatty acids into the circulation (Day, 2002). TNF α also mediates upregulation of hepatic fatty acid translocase, increasing hepatic uptake of fatty acids (Memon *et al.*, 1998).

1.3.3.2 *Increased hepatic fatty acid and triglyceride synthesis*

Insulin resistance leads to hyperinsulinaemia, and consequently to increased fatty acid synthesis by glycolysis and decreased levels of apolipoprotein B-100, reducing triglyceride export. Increased fatty acid synthesis is promoted by the transcription factor, sterol regulatory element-binding protein-1c (SREBP-1c). SREBPs are also activated by hepatitis C viral proteins (section 1.5.2.2). Higher glucose levels lead to increased de novo fatty acid synthesis via carbohydrate response element-binding protein (ChREBP).

1.3.3.3 *Impaired fatty acid mitochondrial β -oxidation*

Saturation of mitochondrial β -oxidation of fatty acids exacerbates hepatic steatosis and is associated with mitochondrial dysfunction and the release of ROS (Angulo, 2002). As mitochondrial oxidative capacity becomes overwhelmed, alternative oxidative pathways are

used, including peroxisomal β -oxidation and endoplasmic reticular ω -oxidation, both of which lead to the increased production of ROS (Reddy, 2001).

1.3.3.4 *Reduced VLDL synthesis and triglyceride secretion*

The rate limiting step for the export of hepatic triglycerides is the lipidation of apolipoprotein B100 by microsomal triglyceride transfer protein (MTP) at the endoplasmic reticulum. Mutations in the gene for MTP lead to the autosomal recessive abetalipoproteinaemia, characterised by severe hepatic steatosis, while a number of drugs which cause hepatic steatosis, including amiodarone, have been shown to have deleterious effects on MTP activity (Anstee and Goldin, 2006). Importantly, HCV core protein has been shown to inhibit this process, providing a direct route to hepatic steatosis in HCV infection (**section 1.5.2.2**).

1.4 Hepatitis C as a relevant model of chronic liver disease

Cohorts of patients with hepatitis C virus (HCV) infection provide an appropriate disease model for the study of chronic liver disease. The natural history of the disease and its histological classification are well established, the prevalence of the disease is high in many countries and effective treatment options are available (Bedossa and Poynard, 1996; Ishak *et al.*, 1995; Poynard *et al.*, 1997). HCV is a single stranded RNA virus from the Flaviviridae family, with an estimated 170 million people infected worldwide (World Health Organisation, 2000). There are a number of HCV genotypes, the most prevalent being genotypes 1 to 4, which are geographically-related and which differ in terms of response to treatment and, controversially, disease progression (Esteban *et al.*, 2008; Zein, 2000). Transfer of blood and blood products is the most common mode of transfer. The median time from infection with HCV to development of cirrhosis has been calculated to be 30 years (Poynard *et al.*, 1997). Within the population, slow, intermediate and rapid fibrosers have been identified, leading to estimates of 33% of those infected developing cirrhosis within 20 years and 31% taking longer than 50 years (Ghany *et al.*, 2003). Viral, host and environmental factors have all been implicated in the variation in progression of fibrosis between individuals (Feld and Liang, 2006). Histologically determined improvement in fibrosis takes between 2 and 3 years, which necessitates follow-up times of this order in longitudinal studies, although a slowing of progression in those responding to treatment compared to those not responding may be detected at earlier timepoints (Dienstag *et al.*, 2003; Poynard *et al.*, 2002).

While many mechanisms of hepatic injury in chronic hepatitis C infection (CHC) are common to other causes of liver disease, some are specific. HCV has a number of receptors on hepatocytes, including CD81 (Stamataki *et al.*, 2008), the LDL receptor and Clq, which are also expressed on stellate cells (Friedman, 2008). Furthermore, hepatitis C viral proteins (non-structural and core) induce stellate cell proliferation and the release of proinflammatory cytokines, which may initiate or augment fibrogenic pathways (Bataller *et al.*, 2004). CHC is also associated with hepatic steatosis, as will be discussed in **section 1.5.2**.

1.5 Hepatitis C and hepatic steatosis

1.5.1 Epidemiology

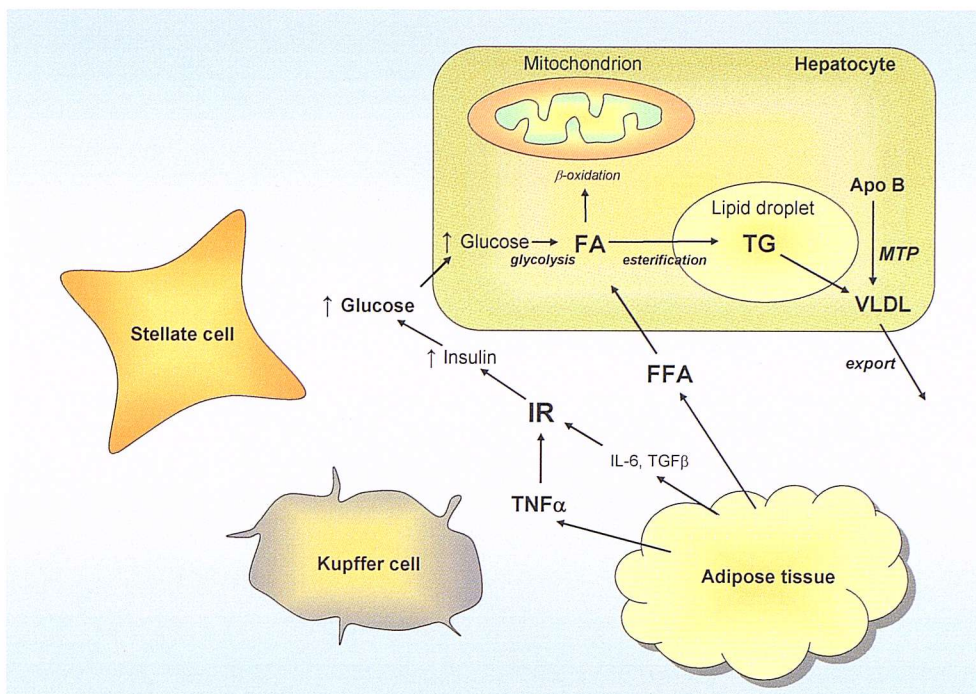
In a review of studies of patients with CHC, the prevalence of steatosis overall ranged from 40-86% (mean around 55%), while the mean prevalence of steatosis in patients with HCV genotype 3 was 73% (Asselah *et al.*, 2006). This prevalence far exceeds that seen in non-infected western populations (Clark *et al.*, 2002; Sanyal, 2002) (**section 1.3.2**).

Steatosis is thus more common in patients with hepatitis C than in the general population. Aetiological factors associated with hepatic steatosis, such as obesity, high alcohol consumption, type-2 diabetes and hyperlipidaemia may also be coexistent in CHC, but the increased prevalence points to specific mechanisms related to the HCV itself, or to the host response to the virus.

1.5.2 Steatosis in HCV

The steatosis score has been shown to correlate with the viral load in patients with genotype 3, but with insulin resistance in genotype 1 (Fartoux *et al.*, 2005). In another study, steatosis in CHC did not correlate with BMI in a cohort of patients with CHC of mixed genotypes, but the grade of steatosis was associated with high visceral fat in patients with genotype 1, but not in those with genotype 3 (Adinolfi *et al.*, 2001). This genotype-related nature suggested that, in patients with CHC, steatosis could have “metabolic” origins, particularly in patients with genotype 1, and “viral” origins, associated most with genotype 3. The relationship between hepatic steatosis, insulin resistance, HCV and liver injury is summarised in **Figure 1-3**.

A



B

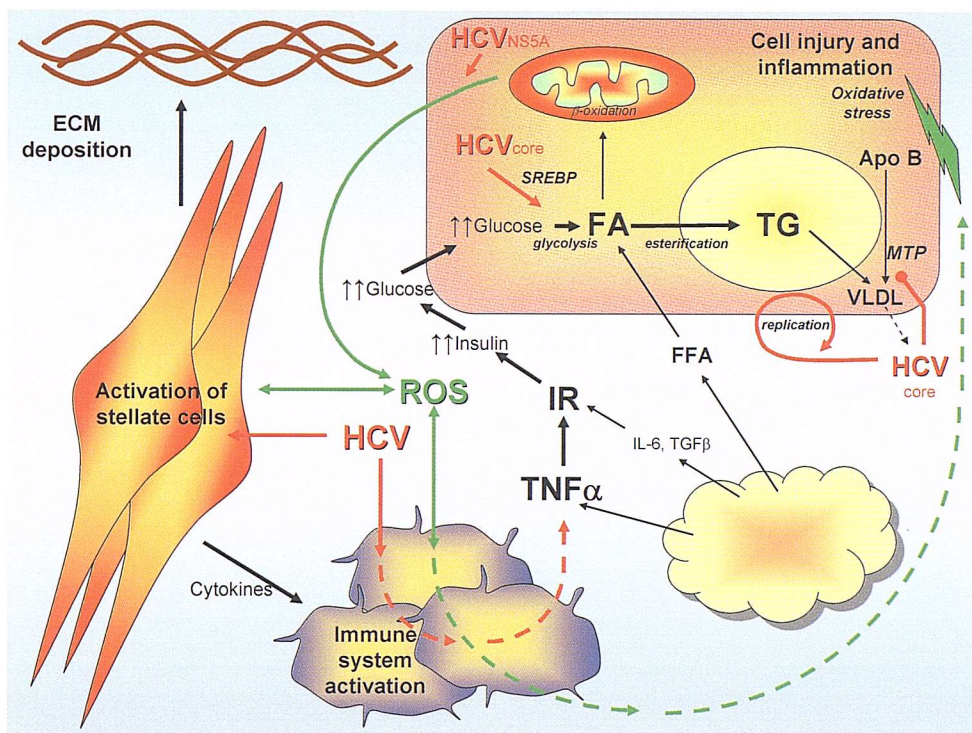


Figure 1-3. Hepatic steatosis, liver injury, insulin resistance and hepatitis C

Panel **A** summarises some mechanisms associated with the development of simple hepatic steatosis. Panel **B** summarises interactions of hepatitis C virus on hepatic steatosis, insulin resistance, oxidative stress and hepatocellular injury. Arrows signify increases or activation; blunt ends, inhibition. Abbreviations: (F)FA, (free) fatty acid; TG, triglyceride; Apo B, apolipoprotein B; MTP, microsomal triglyceride transfer protein; IR, insulin resistance; HCV core, HCV core protein; HCV NS5A, HCV non-structural protein 5A; SREBP, steroid responsive binding protein; ECM, extracellular matrix.

1.5.2.1 “Metabolic” steatosis is associated with insulin resistance in a cytokine-mediated host response to the hepatitis C virus

Epidemiological evidence from a case cohort study suggested that patients with HCV were 12 times more likely to be at high risk of type-2 diabetes (T2DM) (Mehta *et al.*, 2003). This suggested that HCV accelerates the onset in those already at risk of T2DM, but that young people with low BMI were not subject to an increased risk. Accordingly, HCV might be considered a conditional risk factor for T2DM.

A number of studies have documented a relationship between HCV and insulin resistance (Asselah *et al.*, 2006). Patients with HCV have greater insulin resistance than healthy controls (Hui *et al.*, 2003), and the presence of insulin resistance is associated with moderate to severe steatosis (Camma *et al.*, 2006). However such clinical studies are susceptible to confounding by obesity and alcohol use, although, *in vitro* studies have demonstrated specific mechanisms. Mice transgenic for the HCV core protein displayed high serum insulin levels and increased insulin resistance, compared to controls. Feeding a high-fat diet caused diabetes in the transgenic animals but not in controls. Tumour necrosis factor alpha (TNF α) was found to be raised in the transgenic mice and anti-TNF α restored insulin sensitivity (Shintani *et al.*, 2004). Molecular pathways leading to insulin resistance in CHC have recently been reviewed, and include the release of TNF α , IL-6 and TGF β as a consequence of the release of reactive oxygen species (ROS), as part of the Kupffer cell-mediated, pro-inflammatory immune response against the HCV (Sheikh *et al.*, 2008). TNF α modulates adipocytes affecting cytokine, adiponectin and leptin production, which can lead to insulin resistance. TNF α causes down-regulation of glucose transporter gene expression and, consequently, the reduced uptake of glucose into hepatocytes promotes hyperglycaemia and hyperinsulinaemia. Increased IL-6 is part of the virally-induced pro-inflammatory state found in patients with HCV and promotes insulin resistance by means of an acute phase response.

1.5.2.2 “Viral” steatosis is directly mediated by viral proteins

In cell culture, the non-structural gene, NS5A, associates with the endoplasmic reticulum and stimulates mitochondrial ROS production and activating nuclear factor- κ B, upregulating cytokine production (including TNF α). The HCV core protein has been shown to interact with lipid droplets (Barba *et al.*, 1997) and studies have demonstrated that the lipid droplet is involved in the production of infectious viral particles, whereby core protein recruits a replication complex to the lipid droplet-associated membranes (Miyanari *et al.*, 2007). In murine models, HCV core protein was seen to inhibit activity of MTP within the endoplasmic reticulum. MTP stabilises apolipoprotein B (apoB) by lipidation. Lipidated apoB associates with triglycerides to form very low density lipoproteins (VLDL), so decreased MTP activity leads to intracytoplasmic lipid accumulation (Perlemuter *et al.*, 2002). ApoB levels were inversely correlated with the steatosis score and correlated with decreased cholesterol levels in a cohort of male patients with CHC (Serfaty *et al.*, 2001). Furthermore, MTP mRNA expression in liver biopsies from patients with CHC was inversely correlated with the degree of steatosis and with serum insulin levels. In addition, MTP-activity was significantly lower in genotype 3 than in genotype 1 (Mirandola *et al.*, 2006). Cell culture experiments have shown that the production of low density lipoviral particles occurs in the endoplasmic reticulum and is dependent on MTP and apoB, a mechanism closely related to the production of VLDL. These studies demonstrated that HCV coopts VLDL assembly, maturation, degradation and secretory pathways within the cell, providing a possible mechanism for hepatocyte tropism and immune evasion (Gastaminza *et al.*, 2008). Elegant studies by Kapadia and Chisari demonstrated that, in cell lines, HCV RNAs express enzymes involved in cholesterol and fatty acid biosynthesis. These authors showed that the fatty acid content of the cell and the levels of geranylgeranyl pyrophosphate (a post-transcriptionally modified protein originating within the cholesterol biosynthetic pathway) determine HCV RNA replication (Kapadia and Chisari, 2005). Whereas saturated and monounsaturated fatty acids induced HCV RNA replication *in vitro*, polyunsaturated fatty acids (PUFAs) inhibited HCV RNA replication. Furthermore, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (“statins”) have been shown to display antiviral effects

when combined with interferon- α *in vitro* and this effect is reversed by the addition of geranylgeraniol (Ikeda *et al.*, 2006). Core protein may also act indirectly to cause mitochondrial dysfunction by inhibition of peroxisome proliferator-activated receptor alpha (PPAR α). This leads to decreased circulating levels of adiponectin, associated with insulin resistance (Sheikh *et al.*, 2008).

Further studies implicate sterol regulatory binding protein (SREBP), which promotes fatty acid synthesis, in the development of HCV-related steatosis (Mengshol *et al.*, 2007). Cell lines transfected with HCV genotype 1b core protein demonstrated increased SREBP levels and activity (Kim *et al.*, 2007), while HCV (genotype 2a) infection was shown to cause proteolytic cleavage of sterol regulatory binding proteins (SREBP). Core protein and NS4b from genotype 3a were shown to induce proteolytic processing of SREBP. The mechanism for the cleavage and activation of SREBP is thought to arise from oxidative stress and such activation results in the up-regulation of genes involved in lipogenesis and cellular uptake of lipoproteins (Waris *et al.*, 2007).

HCV induces hepatic steatosis through a number of routes and further work is required to elucidate further the mechanisms which may yield targets for future therapy (Mengshol *et al.*, 2007; Sheikh *et al.*, 2008). While a number of direct pathways have been described for the development of “viral” steatosis, “metabolic” steatosis may be considered the result of virus/host interaction and, in particular, the inflammatory response, promoting insulin resistance and hepatic steatosis.

1.5.3 Oxidative stress

A common factor in many pathways for the development of hepatic steatosis and hepatic injury in CHC is the presence of oxidative stress (**Figure 1-3**). Oxidative stress may be defined as an unbalanced increase of ROS with predominantly pro-oxidant activity exerted on different molecules, potentially able to affect their structure and function (Poli, 2000). ROS include

superoxide, hydrogen peroxide and hydroxyl radicals, all of which may directly cause cellular injury and genetic mutations. Lipid cell membrane constituents, particularly PUFA, are the main substrate for peroxidation within the liver and membrane lipid peroxidation end-products, such as covalently-bonded malondialdehyde (MDA) and 4-hydroxy-2,3-nonenal (HNE) adducts, have been detected by immunohistochemistry in chronic hepatitis C, correlating with fibrosis score and histological activity (Paradis *et al.*, 1997b). Although lipid peroxidation products have been documented histologically in a number of chronic liver diseases (Paradis *et al.*, 1997a), higher levels of MDA and activation of glutathione turnover have been demonstrated in patients with hepatitis C than in those with hepatitis B (Farinati *et al.*, 1995). A further study investigated the presence of lipid peroxidation products and antioxidants in a cohort of patients with hepatitis C, finding that levels of peroxidation markers, such as the ratio of oxidized to reduced glutathione and 8-isoprostane were significantly increased, and the levels of antioxidants significantly decreased in patients with both cirrhotic and precirrhotic disease compared to controls (Jain *et al.*, 2002). Oxidative stress is central to a number of mechanisms of hepatic injury and the development of hepatic steatosis.

1.5.3.1 *Oxidative stress is caused by the hepatitis C virus and other insults*

The HCV components, core protein, NS3 and NS5a, can interact with mitochondria and the endoplasmic reticulum to increase the formation of ROS (Okuda *et al.*, 2002) and as described in **section 1.5.2.2**. Ethanol metabolism and the presence of iron overload are also potent sources of ROS, which may exacerbate liver injury.

1.5.3.2 *Oxidative stress is a consequence of immune activation*

Hepatocellular injury, whether by viral, metabolic or toxic insults, leads to immune activation (**section 1.2.4**). HCV causes liver damage by diverse mechanisms including direct cytopathic effects and through an immunological response to virally-infected or necrotic cells. Cytokine release and the activation of macrophages lead to the further production of ROS (Poli, 2000).

1.5.3.3 *Oxidative stress is a cause of steatosis and insulin resistance*

ROS induce cytokine formation by hepatocytes and Kupffer cells (including TNF α , TGF β and IL-6), which leads to increased insulin resistance and, indirectly, to hepatic steatosis, as described in **section 1.5.2.1**.

1.5.3.4 *Oxidative stress is a consequence of steatosis*

Steatosis in hepatocytes is considered a source of ROS, as the presence of excess triglyceride provides “fuel on raging flames” (Koike, 2005). However, hepatic lipid *per se* is not a cause of progressive disease, so other sources of oxidative stress are required (Day and James, 1998).

1.5.3.5 *Oxidative stress is a cause of hepatocellular injury and hepatic fibrosis*

ROS are destructive molecules, causing cellular and genetic damage, which leads to necrosis and immune activation, both potent triggers of oxidative stress. ROS are one of a number of activators of HSCs. It has been shown that activation of HSCs by oxidative stress is a paracrine reaction, mediated by hepatocytes (Bedossa *et al.*, 1994; Svegliati *et al.*, 1998). As liver disease progresses, the disruption of hepatic tissue architecture and sinusoidal function may impair local homeostatic processes and the clearance of toxic metabolites, and cause depletion of antioxidants (Friedman, 2000). Thus, a destructive cycle may be initiated.

1.5.4 Hepatic steatosis, insulin resistance and hepatic fibrosis

A number of cross-sectional studies have found a weak association between the presence of steatosis and a higher degree of fibrosis (Asselah *et al.*, 2006). Conflicting evidence has been presented as to whether the relationship between steatosis and fibrosis is genotype-dependent. In a large European cohort, steatosis was associated with fibrosis only in patients with genotype 3 (Rubbia-Brandt *et al.*, 2004), while in another cohort, fibrosis stage was related to the severity of steatosis in genotype 1 (Patton *et al.*, 2004).

A study comparing factors associated with fibrosis in patients with NAFLD and patients with CHC (genotype 3) found that, while advanced fibrosis was associated with steatosis grade in

NAFLD, steatosis grade was not associated with fibrosis in the cohort of patients with hepatitis C. Insulin resistance was associated with advanced fibrosis in both cohorts (Bugianesi *et al.*, 2006). These observations were extended to patients with CHC, genotypes 1 and 4, in which insulin resistance was independently associated with significant fibrosis and significant fibrosis was associated with severe steatosis (Moucari *et al.*, 2008). In a further study, insulin resistance was an independent predictor of both the severity of fibrosis and the rate of fibrosis progression in CHC (Hui *et al.*, 2003).

1.5.5 Hepatic steatosis in CHC and response to antiviral treatment

The effect of insulin resistance on response to antiviral therapy in patients with CHC, genotypes 2 and 3, has been examined. High insulin resistance was associated with reduced response to treatment (Poustchi *et al.*, 2008). Conversely, in patients with CHC who responded to antiviral therapy, steatosis improved (Castera *et al.*, 2004). Attempts to reduce insulin resistance, such as exercise, weight loss and use of metformin were proposed to lead to improved response to antiviral treatment. Indeed, metformin added to antiviral therapy increased the rapid viral response, although longer term data are awaited (Romero-Gomez *et al.*, 2008a).

1.5.6 Summary

Section 1.5 describes how hepatic steatosis is an important co-factor in chronic liver disease and, in particular, chronic hepatitis C. Steatosis is both a direct and indirect consequence of HCV infection, and a result and a cause of oxidative stress. Epidemiological and clinical studies have shown the co-existence of these conditions to be of importance with respect to the severity and rate of progression of subsequent fibrosis and, particularly in terms of insulin resistance, response to antiviral therapy. Moreover, the intimate relationship between viral replication and cellular lipid metabolism provides the basis for novel approaches to therapy.

It is essential, then, that methods for the clinical assessment of hepatic lipid in chronic liver disease be developed and validated in addition to the development of non-invasive tools for the assessment of hepatic fibrosis.

1.6 Current approaches to the assessment of chronic liver disease

The assessment of patients with chronic liver disease is largely based on assessment of the clinical history and physical examination. Most pre-cirrhotic chronic liver disease is asymptomatic, while clinical signs are subtle and non-specific. Clinical chemistry, so-called “liver function tests” (aminotransferases, alkaline phosphatase, bilirubin and gamma-glutamyl transferase) and haematological indices (cell-counts and the prothrombin time) may be deranged in cirrhotic and pre-cirrhotic disease, but alone, their sensitivity and specificity for the diagnosis and staging of chronic liver disease is limited (Schuppan and Afdhal, 2008).

1.6.1 Liver biopsy is still considered the “gold standard” for disease assessment

Histological assessment of liver biopsy is the mainstay for the diagnosis and staging of chronic liver disease. Epidemiological and pathophysiological data support fibrosis being the hallmark of chronic liver disease and a predictor of outcome (Poynard *et al.*, 1997). Inflammatory and metabolic processes are central to fibrogenesis and information regarding these features is also conveyed by histology. Inherent to liver biopsy is the risk of discomfort, significant morbidity and a small, but important mortality rate, described in **section 1.6.4.1**. As such, it is not optimal for repeated measures, as required by clinical longitudinal interventional studies.

1.6.2 Liver biopsy

Most liver biopsies for the assessment of chronic liver disease are performed percutaneously, by passing the biopsy needle between the ribs into the right lobe of the liver. Guidelines for safe and effective liver biopsy have been published by the British Society of Gastroenterology (Neuberger *et al.*, 2004).

1.6.3 The validity of histological scoring systems

1.6.3.1 *Fibrosis and inflammation*

The interpretation of the histology of liver tissue in chronic liver diseases is based on categorization or scoring of inflammatory features (grade) and fibrosis and architectural disruption (stage) (Standish *et al.*, 2006). The first major scoring system was developed in 1981, and based on the identification of periportal necrosis, intralobular necrosis, portal inflammation and fibrosis, assessed separately and assigned a score (Knodell *et al.*, 1981). This histological activity index was modified subsequently to include extra scores (Ishak *et al.*, 1995). The first three components of the score contribute to the necroinflammatory grade, while the fibrosis score, from 0-6, is based on architectural changes in the pattern and expansion of fibrous bands. Cirrhosis is represented by stage 5 (incomplete cirrhosis) and 6 (probable or definite cirrhosis).

The METAVIR scoring system was developed to look specifically at hepatitis C-related liver disease. Here, the histological activity is based on piecemeal and lobular necrosis, while the fibrosis stage is scored from 0-4, with 4 representing cirrhosis (Bedossa and Poynard, 1996).

All scoring systems are designed to place a numeric value to architectural features. They consist of ordered categorical data representing qualitative and semi-quantitative descriptions, so are not quantitative measures of fibrosis. Studies of liver tissue using digital image analysis have shown that the area of fibrous tissue is not linearly related to the fibrosis score. Moreover, inflammation may cause expansion of fibrous tracts (Standish *et al.*, 2006). Nevertheless, histological fibrosis assessment has been validated by successive clinical studies, making the features clinically relevant (Poynard *et al.*, 1997).

1.6.3.2 *Steatosis*

Scoring systems quantify fat on the basis of visible hepatic lipid droplets within hepatocytes. The grade of steatosis is based on the proportion of hepatocytes containing visible lipid and is expressed semi-quantitatively on a scale of 0-3 (0, <5%; 1, 5% - 33%; 2, >33% - 66%; 3, >66%)

(Brunt *et al.*, 1999; Kleiner *et al.*, 2005). Grading is considered in the context of other histopathological lesions, such as inflammatory infiltration and ballooning of hepatocytes. However, there are considerable limitations to liver biopsy for the quantification of hepatic lipid. Quantification of lipid is based only on visible lipid droplets, so invisible (for example, membrane) lipid is not included. The assessment is only semi-quantitative, being a two-dimensional estimation of the proportion of hepatocytes including lipid, and not considering the volume of droplets. Finally, the composition of the fatty acid components is not assessed, although, using immunohistochemical techniques, the presence of lipid peroxidation products may be determined (Paradis *et al.*, 1997a).

1.6.4 Limitations of liver biopsy

The limitations of liver biopsy have been well-documented but warrant re-iteration in studies that use histology as a reference standard.

1.6.4.1 *Morbidity and mortality*

Percutaneous liver biopsy has a small, but quantifiable, risk of mortality, quoted as between 1 in 1000 and 1 in 10000 patients (Gilmore *et al.*, 1995; Lindor *et al.*, 1996; Perrault *et al.*, 1978). Minor complications, such as post-procedural pain or localised haematoma, occur in between 3% and 30% of cases. More severe complications, including intraperitoneal haemorrhage (requiring transfusion) or perforation of a viscus (including pneumothorax) may occur in 0.3% to 0.6% of cases (Cadranel *et al.*, 2000; Neuberger *et al.*, 2004). Good management of clotting disorders and the appropriate use of the transjugular approach may improve outcome (Neuberger *et al.*, 2004).

1.6.4.2 *Sampling variability*

Percutaneous liver biopsy typically samples less than 1/50000th of the liver, so any heterogeneity of pathological features may lead to sampling variability (Bedossa *et al.*, 2003). Autopsy studies have demonstrated that cirrhosis may be missed on a single pass liver biopsy in between 10% and 30% of cases (Maharaj *et al.*, 1986; Poniachik *et al.*, 1996), while a study

using laparoscopic biopsy of both left and right lobes of the liver found a difference of at least one fibrosis stage between lobes in over 30% of patients (Regev *et al.*, 2002).

The size of the liver biopsy specimen influences sampling variability. Smaller biopsy sizes (in length and breadth) were shown to lead to a lower probability of observing characteristics of more severe diseases and, consequently, led to underestimation of disease severity (Colloredo *et al.*, 2003). The authors recommended that biopsies be longer than 20mm and contain 11 or more complete portal tracts. Nevertheless, even in major centres, as few as 1/6th of samples exceeded this length in a study of over 500 patients (Poynard *et al.*, 2004). A study using digital image analysis of large sections of liver tissue (given a reference score) and “virtual” biopsies of small areas showed that a sample length of 25mm had a 25% error, which reduced to a minimum at 40mm (Bedossa *et al.*, 2003). The type of needle used is also a factor, with Tru-Cut considered superior to Menghini (Colombo *et al.*, 1988). While the size of sample is clearly important for correct interpretation, the number of passes taken to obtain the specimen is correlated with the rate of complications (Tobkes and Nord, 1995).

1.6.4.3 *Subjectivity and inter-observer variation*

Histological scoring systems are designed to be objective and reproducible, but interpretation is still a source of error. Interobserver variability is low for the assessment of fibrosis, but higher for the assessment of activity or inflammation (Goldin *et al.*, 1996). In a study of intra- and inter-observer variability, agreement was better for fibrosis than activity and among experienced pathologists than more junior pathologists (Rousselet *et al.*, 2005). These authors concluded that the experience of the pathologist had more influence on agreement than the characteristics of the biopsy itself.

Histology of liver biopsy specimens is still the mainstay for the definitive diagnosis of liver diseases and for investigation of co-existing pathology. Nevertheless, there are risks inherent in the technique and scoring systems should be interpreted in the knowledge of the limitations.

1.7 Non-invasive techniques

Non-invasive techniques are suited to clinical and longitudinal studies as the risk to patients is limited. In addition, problems related to sampling variability are greatly reduced. Finally, non-invasive techniques may measure clinically relevant parameters not adequately assessed by liver biopsy. Non-invasive tests of chronic liver disease may be broadly divided into serum (or blood) markers and imaging-based technologies. Other approaches include the use of breath tests (Lalazar *et al.*, 2008) and the developing “-omics” technologies, genomics, proteomics (Morra *et al.*, 2007) and metabonomics of biofluids (Lindon *et al.*, 2004). The focus of this thesis is on imaging techniques for the assessment of chronic liver disease and, in particular, ^1H MRS.

1.7.1 Magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) is a non-destructive technique which allows the study of molecular composition and structure. The term encompasses both *in vivo* magnetic resonance spectroscopy (MRS) and *in vitro* MRS at high magnet field strengths (Gadian, 1995).

1.7.2 Proton magnetic resonance spectroscopy

The ^1H MR spectrum represents resonances (peaks) pertaining to protons in differing physico-chemical environments, with the position of each resonance determined by the chemical shift. The area under the peak is proportional to the number of protons contributing to that resonance. The width of the peak is determined, in part, by the T_2 (transverse) relaxation time. Free or unbound protons in smaller, mobile molecules are represented as narrow peaks. Glucose, glycogen subunits, choline-containing compounds, fatty acids and free amino acids are thus all represented, since they are MR-visible. Fats are usually represented by prominent, but relatively broad peaks. The linewidths of the lipid resonances are dependent on molecular mobility and vary according to whether the lipid is free or membrane-bound. Water, being the most abundant compound in most biological tissues leads to a prominent resonance, which, on account of strong hydrogen bonds, is typically a broad peak. Larger macromolecules, such as proteins, and

the amino acids contained therein, are poorly represented, as T_2 relaxation times are very short, leading to very broad and essentially MR-invisible components. In addition, the large broad resonances of fat and water often dominate spectra, obscuring the smaller resonances. These characteristics are important for the interpretation of the spectra. The principles of NMR, where relevant to this study, are considered in more detail in **Chapter 2**.

MRS provides a “snapshot” overview of metabolism in a particular region of interest or sample. Spatial resolution of MRS is of the order of a few cubic centimetres, thus reducing the sampling error inherent in liver biopsy, and while there is low specificity for the analysis of specific pathways, a global assessment may be achieved. These properties make ^1H MR spectroscopy a potentially useful tool for the assessment of chronic liver disease and an adjunct to imaging modalities, for example ultrasound or MRI, which enable assessment of the structural changes associated with chronic liver disease.

1.7.3 Imaging-based modalities assess features of chronic liver disease

Imaging modalities are site-specific; while serum markers may potentially be confounded by extra-hepatic disease, imaging markers are anatomically-defined. Imaging techniques detect a number of signs or physical parameters, which are of relevance to chronic liver disease.

1.7.3.1 *Morphological changes*

The later stages of chronic liver disease are characterized by a number of intra- and extra-hepatic structural changes. A number of these structural changes are manifestations, or consequences of other pathological processes, which will be addressed below.

The cirrhotic liver is typically small, with an uneven border or perimeter. These features are caused by the contraction of thick bands of fibrous tissue interspersed by regenerative nodules. Ultrasound-visible features, such as liver surface nodularity, caudate lobe hypertrophy and the presence of detectable hepatic venous blood flow have been shown to identify those with severe

fibrosis or cirrhosis from a cohort of patients with chronic liver disease, although it is doubtful that the severe fibrosis group alone would be identifiable by such a technique (Colli *et al.*, 2003).

Routine MRI, computed tomography (CT) and ultrasound indices can detect gross morphological changes associated with cirrhosis, but none is sensitive or specific. Moreover, pre-cirrhotic disease is not adequately discriminated (Aube *et al.*, 1999; Awaya *et al.*, 2002; Ito *et al.*, 1999). However, dynamic superparamagnetic iron oxide (SPIO) -enhanced and gadolinium-enhanced MRI demonstrates reticular-nodular patterns, thought to represent septal hepatic fibrosis, the presence of which, taken with an overall (subjective) qualitative assessment, allows the discrimination of moderate and severe from mild fibrosis (**Figure 1-4**) (Aguirre *et al.*, 2006). Digital image processing of unenhanced CT scans has enabled assessment of the distribution of high-attenuation patterning, again presumed to represent fibrosis and distinction between moderate and severe fibrosis (Romero-Gomez *et al.*, 2008b). However, these techniques do not provide a quantitative measure of disease severity, and a number of the techniques remain unvalidated by other centres.

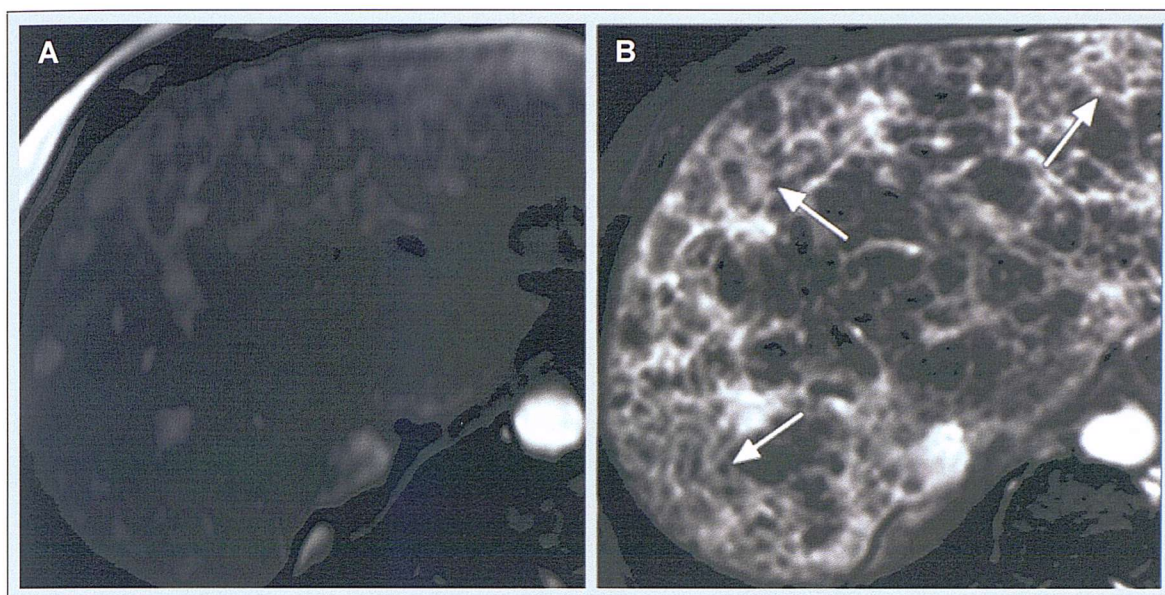


Figure 1-4. Transverse MR images of cirrhotic liver *in vivo*.

A, SPIO-enhanced two-dimensional spoiled gradient echo (SPGR) image with echotime of 2.65ms; *B*, double-enhanced SPGR image at the same level, showing hyperintense reticulations and hypointense nodules (arrows), thought to represent fibrous septal bands surrounding regenerative nodules. Adapted from Aguirre 2006.

Water molecules are tightly bound in the fibrotic extracellular matrix, providing the rationale behind the application of diffusion-weighted MR imaging (DWI) to chronic liver disease. An apparent diffusion coefficient (ADC) is derived, representing proton, and hence water, mobility. A reduced ADC is observed in cirrhosis and with increasing fibrosis stage, and has been interpreted as being due to restriction of water diffusion in fibrotic tissue (Aube *et al.*, 2004; Lewin *et al.*, 2007), or, possibly, by reduced capillary perfusion (Taouli *et al.*, 2007). However, the precise relationship between the ADC and fibrosis is currently unclear.

1.7.3.2 Portal hypertension

Portal hypertension is the cause of much morbidity and mortality associated with chronic liver disease, through development of varices of porto-systemic anastomoses and through activation of vasodilatory pathways and development of ascites and the hepato-renal syndrome. Increased portal pressure is caused by increased intrahepatic resistance to flow, according to Ohm's law. The increased intrahepatic resistance results from both vascular factors and fibrosis (Langer and

Shah, 2006). The structural results of portal hypertension, such as splenomegaly, ascites and the presence of venous collaterals are also readily assessed by conventional imaging techniques, but these features tend to be associated with decompensated cirrhosis and not pre-cirrhotic disease stages.

A number of ultrasound-based studies have aimed to assess portal pressure indirectly as a surrogate for disease severity (Vilgrain, 2001). However ultrasound Doppler indices of portal flow were found not to correlate reliably with increasing severity of disease (Bernatik *et al.*, 2002; Lim *et al.*, 2005a).

Liver stiffness, as discussed below, is a physical parameter that represents the result of a number of biological processes (Cobbold and Taylor-Robinson, 2008). A number of studies have pointed to a relationship between portal hypertension and liver stiffness measurement (LSM). Foucher and colleagues demonstrated a correlation between liver stiffness measurement and splenomegaly, the presence of oesophageal varices and a history of bleeding varices (Foucher *et al.*, 2006). A relationship between LSM and presence of varices has also been described, although evidence for the relationship between LSM and size of varices is mixed (Kazemi *et al.*, 2006; Vizzutti *et al.*, 2007). Vizzutti and colleagues went on to demonstrate correlation between LSM and the hepatic venous pressure gradient (HVPG), particularly at lower HVPG values (<10-12mmHg). This represents a complex relationship, which was less apparent at higher HVPG values (Vizzutti *et al.*, 2007). It has been stated that the “progressive rise in portal pressure...[is]...due mainly to an increase in intrahepatic vascular resistance from the accumulation of fibrillar extracellular matrix” (Lim and Groszmann, 2007). However, increased arterial and portal inflow may contribute directly to the liver stiffness, while haemodynamic changes characteristic of advanced portal hypertension, including extrahepatic haemodynamic changes, may not be detected by changes in liver stiffness.

1.7.3.3 *Intrahepatic vascular changes*

Vascular remodeling is increasingly seen as a pathological feature of chronic liver disease. In the development of fibrosis, obliteration of the small hepatic and portal veins may lead to a congestive hepatopathy, which is exacerbated by a co-existent hyperdynamic circulation (Wanless *et al.*, 1995). This results in inflammation and oxidative stress, both triggers for fibrogenesis (**section 1.5.3**). Intrahepatic vascular remodeling within the fibrotic liver is performed by the contractile HSCs, mediated by changes in levels of nitric oxide (NO), consequent to derangement of endothelial NO synthase. This contributes to high resistance, constricted sinusoidal vessels (Lee *et al.*, 2007). Such mechanisms may also contribute to the development of intrahepatic vascular shunts. Dynamic microbubble contrast-enhanced studies are thought to exploit the intra- and extra-hepatic vascular changes that generate shortening of hepatic vein transit times with increasing disease severity (Albrecht *et al.*, 1999; Lim *et al.*, 2005b). Microvascular remodeling and hepatic congestion may also contribute to changes in water diffusion within a region of interest, measured by DWI (Taouli *et al.*, 2007).

Imaging techniques assess changes in physical properties consequent to vascular alteration. While vascular changes occur with increasing fibrosis, imaging techniques do not assess fibrosis directly, so may be considered surrogate markers in this context.

1.7.3.4 *Liver stiffness*

Liver stiffness measurement by ultrasound-based transient elastography and, more recently, by magnetic resonance elastography (MRE), has proved a promising approach for the assessment of chronic liver disease. Clinically, a cirrhotic liver is hard on palpation, and experimentally, stiffness (elastic modulus) has been correlated with the histopathological fibrosis score in *ex vivo* hepatic samples (Yeh *et al.*, 2002). Biologically, the stiffness of the extracellular matrix has been shown to activate hepatic stellate cells, partly through integrin signalling (Wells, 2008). However, it has been stressed that “biological tissue is a composite material and it is difficult to separate the influence of each component of the tissue on the total in modulus estimates” (Yeh *et al.*, 2002). Therefore, liver stiffness cannot be considered a measure of hepatic fibrosis alone.

The development of transient elastography enabled the rapid acquisition of objective liver stiffness measurements *in vivo* (Sandrin *et al.*, 2003). Multiple regression analysis in early studies demonstrated a relationship between elasticity measurements and fibrosis stage, but not to the disease activity (or necroinflammatory score), nor to the degree of steatosis (Sandrin *et al.*, 2003; Ziol *et al.*, 2005).

A number of studies have confirmed the correlation between liver stiffness and hepatic fibrosis in hepatitis C (Castera *et al.*, 2005; Ziol *et al.*, 2005), and other chronic hepatic conditions (Corpechot *et al.*, 2006; Ganne-Carrie *et al.*, 2006; Yoneda *et al.*, 2007). Recently, meta-analyses and systematic reviews have also been published (Castera *et al.*, 2008; Friedrich-Rust *et al.*, 2008). However, studies have also indicated the presence of co-existing factors that may contribute to liver stiffness, including inflammation (**section 1.7.3.5**), portal hypertension (**section 1.7.3.2**) and, possibly, steatosis (**section 1.7.3.6**).

While ultrasound-based transient elastography measures liver stiffness in a defined region (about 5cm³, right lobe of the liver), MRE has recently been shown to predict fibrosis stage effectively in patients with chronic liver disease, while producing a “map” of liver stiffness, potentially of use to investigate the distribution of pathology (Huwart *et al.*, 2008; Yin *et al.*, 2007).

1.7.3.5 *Inflammation and cell turnover*

The effects of hepatic inflammation have been studied using a number of imaging-based technologies, including MR and ultrasound applications. Hepatic inflammation is associated with cellular inflammatory infiltrate, tissue oedema and hepatocyte swelling. Each of these is likely to affect the physical properties of liver tissue and, as such, can be measured by imaging modalities. These properties include: nuclear relaxation (T₂), assessed by MR techniques; water perfusion and diffusion, as assessed by DWI; liver stiffness; changes in attenuation, assessed by

CT and echogenicity, assessed by B-mode ultrasound. The effect of inflammation on ^{31}P MRS and transient elastography is discussed below.

1.7.3.5.1 *Phosphorus MRS*

Phosphorus (^{31}P) MRS allows the quantification of ^{31}P -containing moieties in tissues. These include energy intermediates (inorganic phosphate, ATP subunits) and phospholipid membrane metabolites (phosphomonoester (PME), thought, broadly, to represent membrane precursors, and phosphodiester (PDE), thought to represent membrane degradation products). Studies in tumour tissue (Ruiz-Cabello and Cohen, 1992), chronic liver disease *in vitro* (Bell *et al.*, 1993; Taylor-Robinson *et al.*, 1995) and *in vivo* (Lim *et al.*, 2003b; Menon *et al.*, 1995) have demonstrated a correlation between the ratios of these resonances and disease state or severity. A study in patients with acute hepatitis A demonstrated an acute rise in PME/PDE, which decreased with resolution of disease (Yamane *et al.*, 1994). Moreover, in patients with hepatitis C, the PME/PDE decreased significantly in those responding to antiviral treatment but did not change in non-responders (Lim *et al.*, 2007).

1.7.3.5.2 *Transient elastography*

The association between liver stiffness and disease activity or necroinflammatory score has been shown by a step-wise increase of LSM with necroinflammatory activity in a cohort of patients with disease of varied aetiology (Fraquelli *et al.*, 2007). Others examined the relationship between LSM and biochemical activity in patients with chronic viral hepatitis. The LSM was lower, stage-for-stage, in those with biochemical remission (as assessed by ALT) than those with a higher ALT (Coco *et al.*, 2007). Recent papers have specifically addressed the effect of hepatic inflammation on LSM. In one, 18 patients without a past history of liver disease, but with acute viral hepatitis, were studied. The LSM at the peak aminotransferase level exceeded 12kPa (the cut-off for prediction of cirrhosis) and furthermore, in all but one subject, the LSM returned to within normal range (below 7kPa). In addition, the LSM correlated with the aminotransferases at onset and with the AST at follow-up (Arena *et al.*, 2008). In another paper, 20 patients with acute hepatitis of varying aetiology (including drug-induced hepatitis) were

studied. In those followed up longitudinally, the aminotransferases returned to a level commensurate with the fibrosis stage at biopsy (Sagir *et al.*, 2008). While it is known that acute hepatitis is associated with an inflammatory infiltrate, tissue oedema and hepatocyte swelling, all of which are likely to affect LSM, there was no histological confirmation of these features in these studies, as liver biopsy was not clinically indicated (Cobbold and Taylor-Robinson, 2008).

1.7.3.6 *Hepatic fat*

Hepatic fat may be assessed using a number of imaging modalities including ultrasound, CT and MR (Mehta *et al.*, 2008).

1.7.3.6.1 *Ultrasound*

Ultrasound is widely used clinically to detect hepatic steatosis, based on the premise that steatosis causes increased echogenicity of the hepatic parenchyma, leading to a brighter image when compared to the cortex of the ipsilateral kidney. Other conditions, such as fibrosis may also lead to increased echogenicity, leading to potential confusion. A review of the non-invasive measurement of fat content found the sensitivity of ultrasound for the detection of steatosis to range from 60% to 94%, while the specificity ranged from 84 to 95% (Mehta *et al.*, 2008). However, histologically-assessed mild steatosis resulted in a sensitivity of just 55% (Ryan *et al.*, 2002). In addition, obesity reduces the accuracy of ultrasound due to technical considerations and increased attenuation of signal caused by subcutaneous fat. Ultrasound performs more poorly for the *quantification* of hepatic lipid, although subjective grading systems, categorizing steatosis into mild, moderate and severe groups have been proposed (Fishbein *et al.*, 2005).

Liver stiffness is thought to be affected by hepatic fat. Substantial differences in cut-off values for cirrhosis have been observed between those with chronic hepatitis and those with alcohol-related chronic liver disease and non-alcoholic fatty liver disease (NAFLD), perhaps representing modification of liver stiffness by coexisting fat (Ganne-Carrie *et al.*, 2006; Yoneda *et al.*, 2007). Liver stiffness was higher in a cohort of patients without evidence of liver disease who had metabolic syndrome, compared to healthy controls (Roulot *et al.*, 2008). Thus, there is

circumstantial evidence that steatosis affects liver stiffness. A recent presentation in which patients were stratified by histological steatosis suggested an increase in liver stiffness with increased steatosis, but these findings need to be corroborated (Lupsor *et al.*, 2008). It appears that LSM is affected by steatosis, but the magnitude of the effect is likely to be smaller than that of other contributing factors, such as fibrosis, inflammation and portal hypertension.

1.7.3.6.2 *Computed Tomography*

CT enables the assessment of hepatic steatosis on the basis of radiographical density (Bydder *et al.*, 1980), although the ability to quantify hepatic lipid is not clear (Park *et al.*, 2006). Ionising radiation associated with CT-scanning confers a small excess risk to subjects, making it less suited for repeated measurements.

1.7.3.6.3 *Proton MRS*

As the ^1H MR spectrum of the liver is dominated by lipid and water resonances, ^1H MRS has been used for the assessment of hepatic fat. The percentage liver fat has been estimated from the number of protons in the lipid and water resonances by calibration with hepatic lipid extracts (Longo *et al.*, 1995). Such measures have been used to assess racial differences in the prevalence of hepatic steatosis and the technique has also been applied in a population of over 2000 participants (Browning *et al.*, 2004; Szczepaniak *et al.*, 2005). Simpler lipid to water resonance ratios have been used to compare hepatic steatosis between obese and lean individuals and have demonstrated a change in intrahepatic lipid in response to dietary intervention (Thomas *et al.*, 2005; Thomas *et al.*, 2006). However, more detailed information on lipid quantitation, composition and the relationship with chronic liver disease remain controversial (**Chapters 4 & 5**).

1.7.4 Serum markers

The development of serum markers is a popular approach for the non-invasive assessment of hepatic fibrosis. Blood tests are quick and acceptable to patients. The results are objective, although there is the possibility of the presence of confounding factors from extrahepatic

disease. These have been reviewed extensively elsewhere (Parkes *et al.*, 2006b; Rockey and Bissell, 2006; Shaheen and Myers, 2007). Serum markers may broadly be divided into “indirect” and “direct” markers, single tests and panels. Indirect markers are markers of liver function, which reflect liver fibrosis, while direct markers include serum ECM components and intermediates of fibrogenesis (Manning and Afdhal, 2008). A selection of serum markers are described in **Table 1-1**. The APRI (aspartate aminotransferase (AST) to platelet ratio index) and the ELF (“Enhanced” Liver Fibrosis) score will be investigated further in **Chapter 6**.

Table 1-1. Examples of serum markers for the assessment of hepatic fibrosis

Name	Constituents	Reference
<i>Indirect markers</i>		
APRI	AST, platelets	(Wai <i>et al.</i> , 2003)
Forns	platelets, cholesterol, γ GT, age	(Forns <i>et al.</i> , 2002)
FibroTest	α 2 macroglobulin, α 2 and γ globulin, total bilirubin, apolipoprotein A ₁ , γ GT	(Imbert-Bismut <i>et al.</i> , 2001)
<i>Direct markers</i>		
HA	Hyaluronic acid	(Guechot <i>et al.</i> , 1996)
ELF	PIIINP, HA, TIMP-1, (age)	(Rosenberg <i>et al.</i> , 2004)
FibroSpect	HA, TIMP-1, α 2 macroglobulin	(Patel <i>et al.</i> , 2004)

Abbreviations: APRI, aspartate aminotransferase (AST) to platelet ratio index; gGT, γ -glutamyl transferase; HA, hyaluronic acid; PIIINP, amino terminal of procollagenase III; TIMP-1, tissue inhibitor of matrix metalloproteinase 1.

1.8 Rationale for the project

This introduction describes how chronic liver disease results in a major disease burden globally. Chronic liver disease arises from numerous aetiologies including viral hepatitis, which have a high prevalence worldwide, and NALFD, which is an increasing problem in westernised societies, associated with the metabolic syndrome of obesity, insulin resistance and dyslipidaemia. The pathogenesis of chronic liver disease has been reviewed in the context of viral hepatitis and hepatic steatosis. Fibrogenesis, the development of the prognostically key pathological lesion, fibrosis, is a complex, multi-step process involving multiple cellular and molecular pathways. Increasing numbers of potential targets for therapy are being uncovered, with the possibility of slowing the progression of disease and even the reversal and resolution of fibrosis. There is increasing recognition that hepatic steatosis is a pathologically-relevant cofactor in the development of a number of chronic liver diseases. In particular, hepatic steatosis is intimately related to the pathogenesis of chronic hepatitis C. Currently, however, assessment of hepatic fat by liver biopsy is limited, while imaging techniques can assess fat quantity and distribution, but not lipid types or composition.

The current gold-standard for the assessment of chronic liver disease, liver biopsy, has limitations and engenders risk to patients. As such, the lack of suitable techniques for the safe, reliable and biologically-relevant assessment of chronic liver disease represents a major barrier to the development of novel therapies. Non-invasive techniques for the diagnosis and staging of chronic liver disease are required and approaches may be broadly divided into the development of serum markers and imaging techniques. Imaging techniques are able to assess a number of pathologically-relevant features of chronic liver disease and form the focus of this thesis.

Proton magnetic resonance spectroscopy is a non-destructive, non-ionising technique, which provides information about the physico-chemical environment of protons within a sample or an anatomical region of interest. ^1H MRS *in vivo* has been used to quantify hepatic lipid in a

number of studies, but, its potential to provide more detailed biological information has received little attention.

For clarity in this thesis, *in vivo* studies on the clinical MR scanner will be denoted “MRS”, to distinguish them from *in vitro* studies, to be denoted “NMR”. ^1H NMR spectroscopy of intact tissue at high field strength *in vitro* enables the generation of high-resolution MR spectra, which provide detailed information on tissue metabolism, in particular, lipid, carbohydrate and amino-acid composition and relative concentrations. Application of this technique to liver tissue in examples of chronic liver disease may provide a framework to inform the interpretation of the lower resolution MRS, obtained *in vivo* at lower field strength. This application is available on most clinical MRI scanners, and MRS sequences may be added to other MR imaging protocols. As such, ^1H MRS may provide biologically and clinically-relevant information related to chronic liver disease.

1.9 Hypotheses

1. Characteristic metabolic perturbations in models of chronic liver disease are detectable using *in vitro* ^1H NMR spectroscopy
2. High resolution ^1H NMR spectroscopy of intact human liver tissue informs *in vivo* ^1H MRS studies
3. *In vivo* ^1H MRS findings complement other non-invasive techniques for the assessment of chronic liver disease

1.10 Aims

The overall aim of this thesis is to apply and validate ^1H MRS as a non-invasive technique for the assessment of chronic liver disease.

In order to achieve this aim, there are a number of subsidiary aims:

1. To optimise the experimental conditions for ^1H NMR spectroscopy of intact liver tissue
2. To examine the differences in the ^1H NMR spectrum from murine models of NAFLD *in vitro*
3. To investigate metabolic differences as assessed by ^1H NMR spectroscopy with reference to histological features in murine models of NAFLD and human liver biopsy samples from patients with chronic hepatitis C
4. To apply *in vitro* NMR spectroscopy findings to inform MR spectral analysis *in vivo*
5. To compare the performance of imaging-based and serum markers of chronic liver disease (APRI, ELF, TE, HTT), head-to-head and in combination compared to histological assessment of liver biopsy in patients with CHC.
6. To validate *in vivo* ^1H MRS for the assessment of CHC in the context of other non-invasive markers of chronic liver disease.

1.11 Structure of the thesis

The structure of the thesis is divided broadly into three parts:

Development of ^1H NMR spectroscopy of intact liver tissue: in **Chapter 2** the parameters affecting acquisition of high-quality NMR spectroscopy data are considered and protocols are developed for robust and stable analysis of intact tissue. In **Chapter 3**, the technique is applied to murine models of NAFLD. Multivariate pattern-recognition tools are used to separate groups and to pinpoint metabolic differences. Disease-relevant MR indices are derived and tested.

Translation of ^1H NMR spectroscopy to the clinical setting: in **Chapter 4**, the techniques previously developed are employed for the analysis of human liver tissue from patients with chronic hepatitis C. In **Chapter 5**, disease-related MR indices, in particular, fatty acid functional group resonances, are applied to ^1H MR spectra obtained at lower field strength, non-invasively, *in vivo*. The indices developed *in vitro* are then validated in a separate cohort of patients with chronic hepatitis C *in vivo*.

Evaluation of ^1H MRS for the assessment of chronic liver disease: In **Chapter 6**, four currently-available non-invasive techniques for the assessment of chronic liver disease are compared, head-to-head and in combination, to liver biopsy in a cohort of patients with chronic hepatitis C to establish the most accurate and reliable markers for assessment of fibrosis stage and inflammatory grade. In **Chapter 7**, the ^1H MRS indices developed during the course of this research are compared, quantitatively and qualitatively to the best performing markers as assessed in **Chapter 6**. In **Chapter 8**, work that would improve or validate the technique further is discussed. Finally, in **Chapter 9**, conclusions from this thesis are drawn and the potential place of ^1H MRS in clinical and research protocols is considered.

2 TECHNIQUE OPTIMISATION STUDIES

2.1 Abstract

NMR spectroscopy provides information on the chemical composition of samples based on differences in the magnetic field experienced by nuclei within the sample. The resonances (or peaks) proportionately represent the nuclei contributing to particular chemical functional groups within the sample. The spectral resolution obtained from solid samples is improved by spinning the sample at 54.7° to the magnetic field.

The aims of this chapter were to: 1) define the physical and technical variables that affect the quality of the NMR spectra and the information yielded; 2) optimise the experimental conditions for spectral acquisition; 3) discuss approaches to spectral analysis.

NMR spectroscopy studies of intact liver tissue were carried out at 11.7T. Snap-frozen murine liver tissue was examined. The effect of sealing cell size, acquisition temperature and experimental duration on spectral quality and the resultant metabolic profile were assessed. Using optimised techniques, high resolution ^1H magic angle spinning NMR spectra were produced and the resonances assigned with reference to the literature. Larger sample sizes and smaller cells yielded a higher signal-to-noise ratio. Higher acquisition temperature led to an increased signal-to-noise ratio, but led to irreversible spectral changes with increasing experimental duration.

2.2 Principles of magnetic resonance spectroscopy (Gadian, 1995)

NMR spectroscopy relies on the subtle differences in the magnetic field strength experienced by specific nuclei within different chemical environments or structures, to cause small differences in the nuclear energy levels. Excitation by radiofrequency energy at a frequency equalling that of the precessional frequency of the nucleus causes the nucleus to change energy level. Upon relaxation, energy at the precessional frequency is detected by the receiver coil.

2.2.1 The NMR phenomenon

The nuclei of some atoms may be considered to rotate around an axis; a phenomenon termed ‘spin’. Rotation provides angular momentum to the charged particle, producing a local magnetic field. These nuclei precess around an axis parallel to an externally applied magnetic field. The angular frequency or precession frequency (ω_0) is the rate of rotation in radians per second and is described by the Larmor equation 2-A:

Equation 2-A

$$\omega_0 = \gamma B_0$$

where γ is the magnetogyric ratio, a property of a particular nucleus within a particular environment, and B_0 is the applied magnetic field.

Accordingly, the angular frequency is proportional to the applied magnetic field and equal to the resonant frequency of a nucleus.

Nuclear dipoles may lie parallel or anti-parallel to B_0 but the net alignment according to the Boltzman distribution determines the resultant magnetism (**Figure 2-1**). When a pulse of radiofrequency energy is applied at the resonant frequency, there is excitation of the nuclei from

equilibrium. The population difference between energy states is small, accounting for the low sensitivity of NMR and the requirement for application of high magnetic fields.

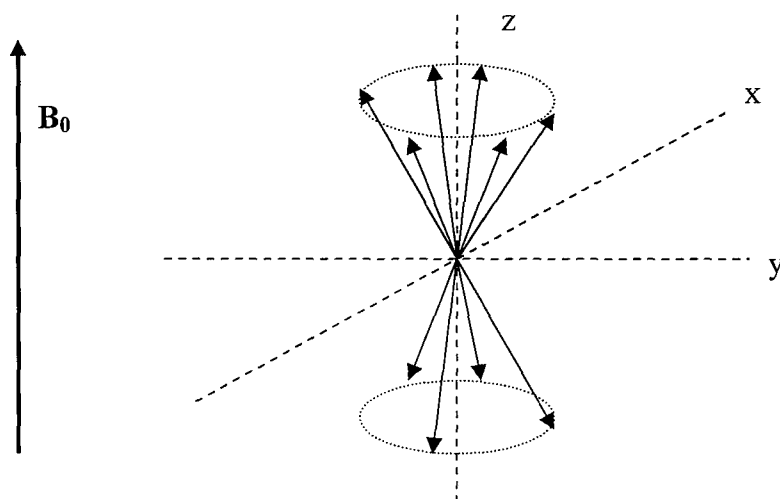


Figure 2-1. Nuclear alignment in an applied magnetic field.

The static applied magnetic field, B_0 runs parallel to the z axis. The nuclei may align with the field (the low energy state) or against the field (the high energy state).

The resonant frequency induces an electric signal in the receiver coil, which may be amplified and detected as a radiofrequency signal known as a free induction decay (FID). The amplitude of the signal decays with time as relaxation occurs towards the resting state.

2.2.2 Nuclear relaxation

On relaxation of nuclei following the application of an RF pulse, there is dephasing of nuclear orientations with transfer of energy between nuclear spins, leading to a reduction in the resultant signal. This relaxation is described by the transverse, or “spin-spin”, relaxation time, T_2 . Longitudinal, or “spin-lattice” relaxation, T_1 , describes energy transfer between the nuclear spins and their molecular framework, the lattice.

A number of mechanisms exist to explain the relaxation phenomena. A dipole-dipole interaction refers to the moment that exists between two or more dipoles or charged particles. The dipole-dipole interaction is strongly distance dependant and a dominant mechanism in molecules with a spin of $\frac{1}{2}$. For nuclei with spin $>\frac{1}{2}$, transfer of energy to the orbiting electrons provides an efficient relaxation mechanism, the quadrupole interaction, which makes relaxation almost instantaneous and the resulting signal difficult to detect. The state of the immediate environment of a nucleus is an important contributor to the relaxation properties. Thermal motion in liquids and solutions contributes to relaxation by the transfer of thermal energy. Nuclei may be shielded from the applied magnetic field and the degree of shielding may be dependent on the orientation of a molecule (hence bonds) with respect to the applied magnetic field, termed *chemical shift anisotropy*. If the molecules are freely mobile, the fluctuation of orientation in a population results in cancellation of the chemical shift difference overall. However in solids, this will result in line broadening.

2.2.3 Chemical shift

The chemical and physical environment of a nucleus alters the magnetic field experienced locally by an individual nucleus. Movement of electrons around a nucleus and the spin of charged particles create small local magnetic fields proportional to, but opposing, the applied field. This has the effect of shielding or screening the nucleus from the full applied magnetic field. Different atoms (or protons in the case of ^1H MR spectroscopy) within a molecule, different types of molecule and their chemical environment may therefore be distinguished. As the magnetic fields produced by electrons are very small compared to the $\mathbf{B}_0$, high applied magnetic fields are required to separate specific entities. In addition to the effects of electrons, local magnetic field inhomogeneities will also influence the locally-experienced magnetic field.

The resonant frequency of a particular nucleus is thus altered for a given applied magnetic field. This leads to alteration of the frequency of the induced signal, so information regarding the

interaction between radiofrequency energy and matter is conveyed; the *spectrum*. The alteration in frequency of a given nucleus from a reference frequency is termed *chemical shift*. The chemical shift is compared to a reference that may be integral to (internal), or separate (external) from, the sample. The chemical shift is expressed in parts per million (ppm), a dimensionless unit, by dividing the frequency difference between the nucleus of interest and the reference by the frequency of the reference and multiplying by a million.

2.2.3.1 *Spin-spin (J) coupling*

The spectral pattern is complicated by the magnetic interaction between neighbouring nuclei within a molecule. This leads to a pattern of resonance-splitting that aids the identification of compounds within a studied sample. The separation of the split peaks is independent of the applied magnetic field.

2.2.3.2 *Signal generation*

If a pulse of energy is applied, the susceptible nuclei are aligned according to the applied field and their oscillations are ‘in phase’. On cessation of the pulse, relaxation of the spin orientations towards the resting state occurs. However, on account of the slight differences in frequency between nuclei caused by the interactions described above, there is ‘dephasing’ of the signal with time. Dephasing leads to increasing destructive interference between emitted radiofrequency energy and therefore decay of the signal. The greater the difference in phase between spins, the faster the decay. The faster the decay of the signal, the shorter the T_2 and the broader the line width becomes.

2.2.3.3 *Quantification*

The raw signal produced when a sample relaxes completely following excitation is quantified by the area under the curve (or amplitude) of the resonances in the frequency domain, and is proportional to the number of nuclei contributing to the signal. A metabolite within a sample may be quantified relative to a reference compound of known concentration or relative to other metabolites within the sample.

2.3 Magic angle spinning nuclear magnetic resonance spectroscopy

2.3.1 Rationale

In **section 2.2.2**, mechanisms of NMR relaxation were described. Dipole-dipole interactions are spatially orientated with respect to the applied magnetic field. In a solution or liquid, free molecular motion causes those orientations to average out, but in solids and semi-solid samples, there are constraints to the orientation of molecular interactions, leading to differences in the relaxation of nuclei within the same chemical environment, and thus causing line-broadening. By spinning the sample at 54.7° , the *magic angle*, spatial differences in relaxation properties are averaged to zero (Andrew *et al.*, 1959). There are magnetic field inhomogeneities at the molecular level caused by the influence of neighbouring nuclei on the electron shielding of a nucleus- chemical shift anisotropy, and macroscopically, magnetic field inhomogeneity may result from gross physical inhomogeneities in a sample, as might be observed in a tissue sample containing membranes, fluids and connective tissue. These spatially-related causes of differences in the magnetic field strength and thus precessional frequency, leading to line-broadening, are averaged out by spinning the sample at the magic angle.

2.3.2 Application to biological tissue samples

The benefits of magic angle spinning (MAS) NMR make it a useful technique for the analysis of tissue samples. MAS NMR allows simultaneous analysis of lipid and aqueous compartments of intact solid tissue, resembling *in vivo* findings more closely than tissue extracts. In addition, MAS NMR spectroscopy requires a smaller mass of tissue for analysis compared to tissue extracts (Martinez-Granados *et al.*, 2006) and enables histological evaluation of the sample following analysis (Dave *et al.*, 2004). MAS NMR spectroscopy has been applied to metabolic profiling in liver and other tissues from animal models and from humans, demonstrating identification of numerous metabolites and provision of a snap-shot metabolic profile.

Since the first studies using ^1H MAS NMR, applied to lymph node tissue (Cheng *et al.*, 1996), the technique has been applied to the study of brain (Cheng *et al.*, 1997), cardiac (Griffin *et al.*, 2001; Jones *et al.*, 2005), prostate (Cheng *et al.*, 2001), breast (Sitter *et al.*, 2002), cervix (Mahon *et al.*, 2004b), pancreas (Fang *et al.*, 2007) and liver (Yang *et al.*, 2007) tissue. Malignant tissue has been studied in a number of settings as the metabolic abnormalities are substantial. More recently, ^1H MAS NMR has been used, together with other metabolic and genetic profiling techniques, in toxicology studies (Waters *et al.*, 2006).

2.3.3 Application to the study of liver disease in man

Just three published studies have used ^1H MAS NMR spectroscopy to examine intact liver tissue in man (Duarte *et al.*, 2005; Martinez-Granados *et al.*, 2006; Yang *et al.*, 2007). Each has examined a small number of samples and sample provenance is varied, so disease-specific conclusions are unclear.

Duarte and colleagues performed the first published study of ^1H MAS NMR spectroscopy of intact liver tissue in man. The main purpose was to outline the metabolic profile of such tissue and to assign the resultant resonances. The clinical model was of donor liver tissue from six subjects for transplantation before retrieval, following storage and following transplantation into the recipient (a total of 18 samples). The main conclusions were a “proof-of-principle” of ^1H MAS NMR spectroscopy as a potential screening tool for the assessment of the metabolic status of donor liver. Their biological conclusions were of identification of University of Wisconsin solution in biopsies during storage and of decreased glycerophosphocholine (GPC) levels throughout the transplant process, associated with presumed increased cell turnover (Duarte *et al.*, 2005).

Martinez-Granados and colleagues applied ^1H MAS NMR spectroscopy to intact liver biopsy samples from 16 patients with chronic liver diseases. 10 of these samples were of chronic hepatitis (aetiology, stage and grade unspecified), three were of cirrhotic liver (pathological

features and aetiology unspecified), two were from “non-pathological” liver adjacent to metastases (of unspecified origin) and one was from “non-pathological” liver, harvested from an elderly woman at autopsy, 24hr *post-mortem*. The stated aim of this study was the applicability to clinical studies of ^1H MAS NMR biochemical profile determination in intact liver tissue by needle biopsy. Intensity ratios of resonances were used to derive indices separating “non-pathological” tissue, “hepatitis” and “cirrhosis”, including glucose:lipid, undefined fatty acid functional group ratios and alanine:lysine. As the disease classification was unclear, numbers were small, “non-pathological” tissue was unlikely to be metabolically normal and the stated ratios without known biological relevance, the conclusions of the study were again a statement of feasibility, rather than biologically plausible or clinically-useful information. Of importance, however, was their study of tissue degradation with time. They concluded that data acquisition at low-temperature led to negligible tissue degradation over a 4hr period (Martinez-Granados *et al.*, 2006).

Yang and colleagues applied the technique to biopsy samples from 17 patients with hepatocellular carcinoma (HCC), taking samples from 17 tumours and 14 adjacent areas of “non-involved” tissue. They aimed to assess applicability of the technique, to classify tumours according to metabolic differences and to assess the effect of co-existent cirrhosis on the metabolic profiles of HCC tissue. They applied principal components analysis to the multivariate data, separating tumour from adjacent tissue and “high-grade” from “low-grade” HCC. The metabolites thought to contribute to differences between tumour and adjacent tissue included increased bile acids, choline-related compounds, and a number of amino acids and decreased glucose, glycogen and lipids in tumour tissue. Moreover, they observed increased lactate and amino acids in high-grade compared to low-grade tumours. However, the authors did not specify how resonances were assigned. This study constituted application of ^1H MAS NMR to a clinical problem, with biological plausibility of some of the results. However, metabolic changes in tumour tissue are likely to be gross, on account of tissue hypoxia, necrosis and cellular content (Yang *et al.*, 2007).

These three studies demonstrate the potential applicability of ^1H MAS NMR to the clinical setting. Challenges shared by these studies include the appropriate classification of tissue samples used, the choice of experimental conditions and the biological relevance of the metabolic perturbations observed.

2.3.3.1 *Extracts*

Prior to the application of ^1H MAS NMR to the analysis of biological samples, lipid and aqueous extracts enabled tissues to be analysed in the solution state. By necessity, this is a destructive technique, but separation of the aqueous phase enables the analysis of water-soluble metabolites, without interference from larger co-resonant lipid peaks. Extract studies typically require >250mg of tissue and concentrations of metabolites may be affected by their solubility in the chosen solvent (Martinez-Granados *et al.*, 2006; Maxwell *et al.*, 1998).

2.3.4 Technical considerations

^1H MAS NMR of intact tissue is a relatively new technology. The physical parameters and technical considerations vary between studies. The physical forces applied to the sample, spatial variation in radiofrequency (RF) energy and magnetic field across the sample all complicate the acquisition of high quality MR spectra of tissue, compared to biofluids. Optimal sequences and experimental conditions have not been established and depend on the hardware available and the information required from the study. **Table 2-1** summarises some of the technical and physical variables from three studies, highlighting the lack of current consensus.

Table 2-1. Published methods for ^1H MAS NMR of intact liver tissue

	Bollard et al. 2000	Duarte et al. 2005	Martinez-Granados et al. 2006
System	Bruker, 400MHz	Bruker, 400MHz	Bruker, 500MHz
Tissue type	Rat liver	Human donor liver (pre-retrieval; post-storage; post-transplant)	Liver needle-biopsy samples, CLD of varying aetiology,
Sample size	c. 8mg	$19.5 \pm 1.0\text{mg}$	0.5mg – 12mg
Sample prep	Perfused with 0.9% NaCl with D_2O	“Immediately” snap-frozen in liquid N_2	“Cooled immediately”
Sample storage	Snap-frozen in liquid N_2 , then stored at -70°C	Stored at -70°C, then washed with D_2O saline (0.9%)	Stored at -80°C until use
Insert (sealing cell) type	Not specified	4mm diameter zirconia rotors with “top inserts”, 15 μl final volume	4mm diameter zirconia rotors with spherical insert, topped up with D_2O to 12 μl final volume
Acquisition temperature	30°C	25°C	0°C, sample temperature calculated as 4°C
Spin speed	6200Hz	4000Hz	4000Hz
Sequences used	Presat/ NOESYPR1D CPMG TOCSY JRES HMQC	Presat CPMG TOCSY JRES HMQC	Presat/ 1DNOESY CPMG TOCSY HSQC
Number of scans (“transients”)	128 for presat 256 for CPMG	128 for presat 256 for CPMG	400 - 1024
Echo time (for spin-echo sequences)	80ms	120ms	36ms

Abbreviations: CLD, chronic liver disease; D_2O , deuterated water; presat, fully-relaxed pulse and collect spectrum with water presaturation; NOESYPR1D, one-dimensional nuclear Overhauser effect spectroscopy with water presaturation; CPMG, Carr-Purcell-Meiboom-Gill sequence (spin-echo); TOCSY, total correlation spectroscopy; JRES, J-resolved spectra; HMQC, heteronuclear multiple quantum correlation; HSQC, heteronuclear single quantum correlation.

2.4 Aims

The experiments in this chapter were performed to optimise experimental conditions in order to obtain high quality, high resolution ^1H MAS NMR spectra *in vitro* on the system to be used for subsequent experiments, by testing physical and NMR parameters.

2.5 Hypotheses

Using ^1H MAS NMR of liver tissue, it was hypothesised that:

- a. Increased sample size increases the signal-to-noise ratio
- b. Decreased chamber size increases spectral resolution
- c. Increased temperature of acquisition increases spectral resolution but increases rate of sample degradation
- d. Longer duration of acquisition increases signal-to-noise, but also increase sample degradation

2.6 Methods

A number of physical and NMR parameters affect the quality of data obtained by ^1H MAS NMR spectroscopy. For experiments contributing to this thesis; parameters were defined on the basis of evidence from the literature. Where published evidence was lacking, experiments were performed to establish the optimum conditions for data acquisition.

2.6.1 Equipment

Proton MAS NMR experiments were carried out using a MAS probe (Doty Scientific Inc., Columbia, South Carolina, USA), interfaced with a JEOL Eclipse 500+ MR spectrometer (JEOL (UK) Ltd, Welwyn Garden City, UK) and a 11.7T superconducting magnet (Oxford Instruments, Oxford UK). Air temperature was detected by thermocouple and spin rate by an optical spin counter. A temperature control system was developed whereby water-depleted compressed nitrogen gas was passed through a heat exchange coil in liquid nitrogen prior to entering the probe and spinning the rotor. A heater and thermocouple were used to keep the temperature to the required settings.

2.6.2 Sample provenance

2.6.2.1 *Sample acquisition*

Murine liver samples were obtained from mice culled by exsanguination under terminal anaesthesia, minimising residual blood in the sample.

2.6.2.2 *Sample storage*

All liver tissue samples were placed in a polypropylene tube and snap-frozen in liquid nitrogen immediately after acquisition and typically within 2 minutes of harvest in order to minimise sample degradation. Samples were transferred to a freezer at -80°C for longer term storage.

2.6.2.3 *Sample preparation*

Samples were transferred to dry ice prior to preparation. Samples were thawed to the extent that they could be cut with a scalpel (typically up to 30s) and the remainder immediately placed back onto dry ice. The samples were inserted into pre-cooled, pre-weighed XC4 Kel-F sealing cells with Teflon plugs (Doty Scientific Inc., Columbia, South Carolina, USA) and the samples and cells weighed. The sample mass was determined by deduction. The prepared cells were then kept on dry ice prior to analysis. The filled cells were placed in pre-cooled silicon nitride rotor tubes with aurum finned caps (Doty Scientific Inc., Columbia, South Carolina, USA) prior to insertion into the probe.

2.6.3 NMR parameters

2.6.3.1 *Choice of sequences*

The ^1H MAS NMR spectrum of liver tissue is dominated by water and lipid resonances. Choline-related compounds, glucose and glycogen resonances are prominent, while a number of amino acid resonances are also detectable. NMR sequences were chosen to enable accurate assessment of all components.

To assess the abundance of major lipid resonances compared to water content of samples, a fully-relaxed pulse and collect sequence was used (**Figure 2-2**). To compare the lipid components and choline-related compounds, assess measures of lipid saturation, and for used in multivariate analysis, a fully-relaxed pulse and collect spectrum with water presaturation (water suppression) was used (**Figure 2-3**). This sequence enabled simultaneous examination of lipid and other metabolite components without systematic suppression of either component. To investigate smaller metabolites, including the individual components of the choline region, a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with water presaturation was used (**Figure 2-4**).

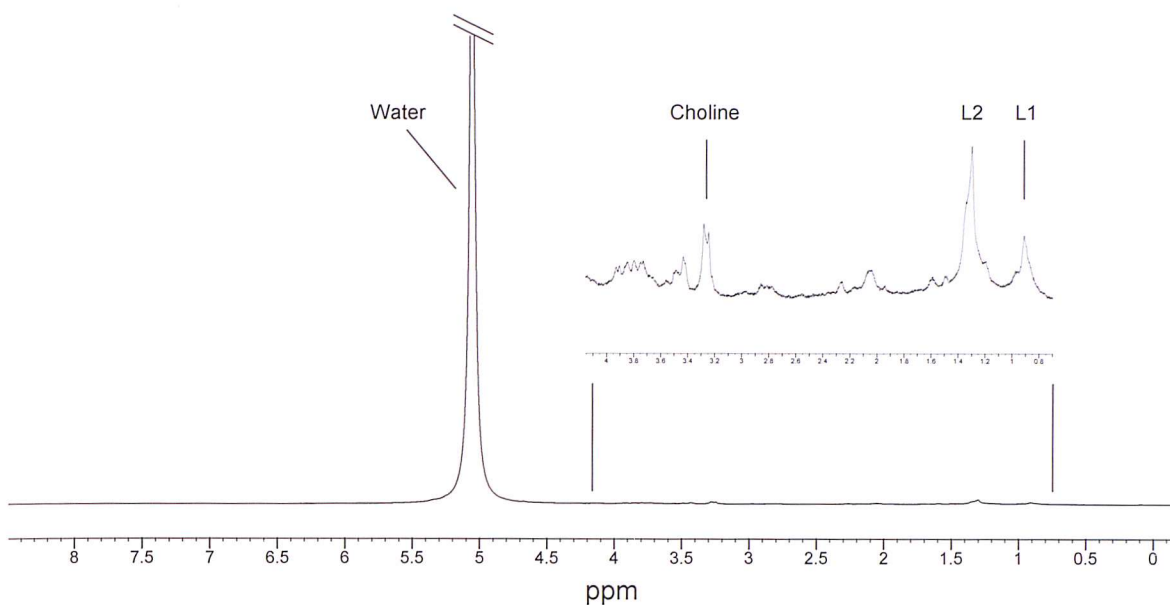


Figure 2-2. Pulse and collect spectrum without water suppression.

A fully relaxed pulse and collect spectrum from normal murine liver shows a spectrum dominated by water. Magnification on the y-axis clearly shows the presence of the main spectral peaks, such as L1, methyl functional group and L2, methylene and the choline region.

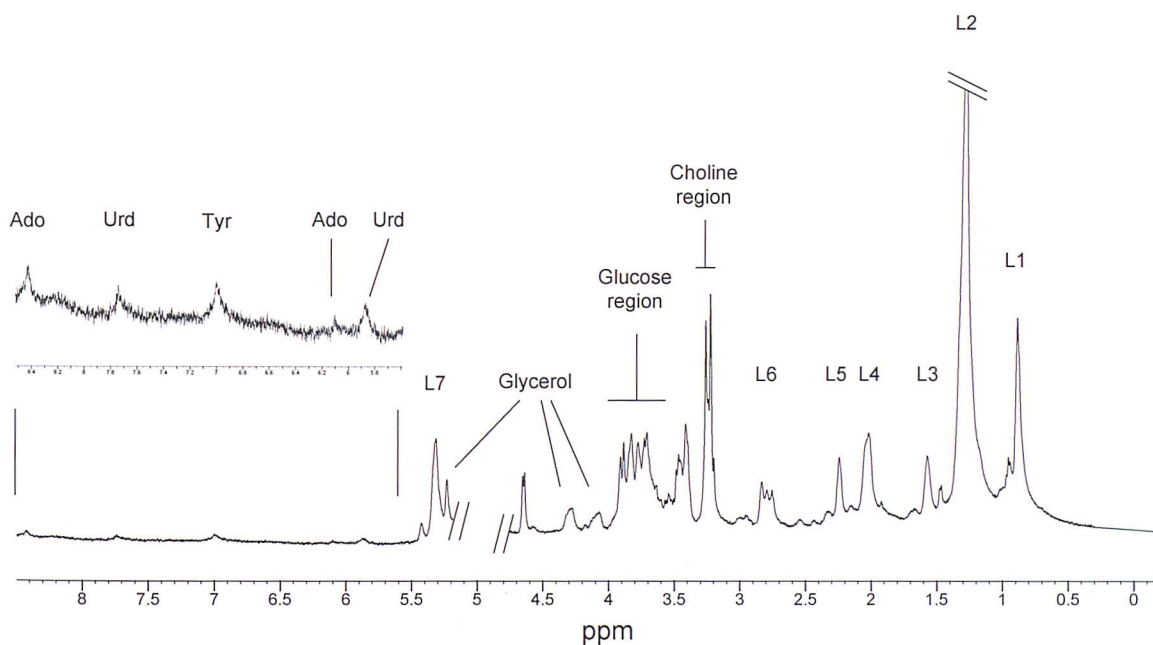


Figure 2-3. Pulse and collect spectrum with water suppression.

The lipid spectrum; a fully relaxed water-presaturation spectrum demonstrates all the main lipid functional groups. The components of the choline and glucose/glycogen region may also be seen. On magnification of the region from δ 5.5-8.5ppm, a number of amino acid resonances are visible. The residual water peak has been removed and the L2 peak truncated.

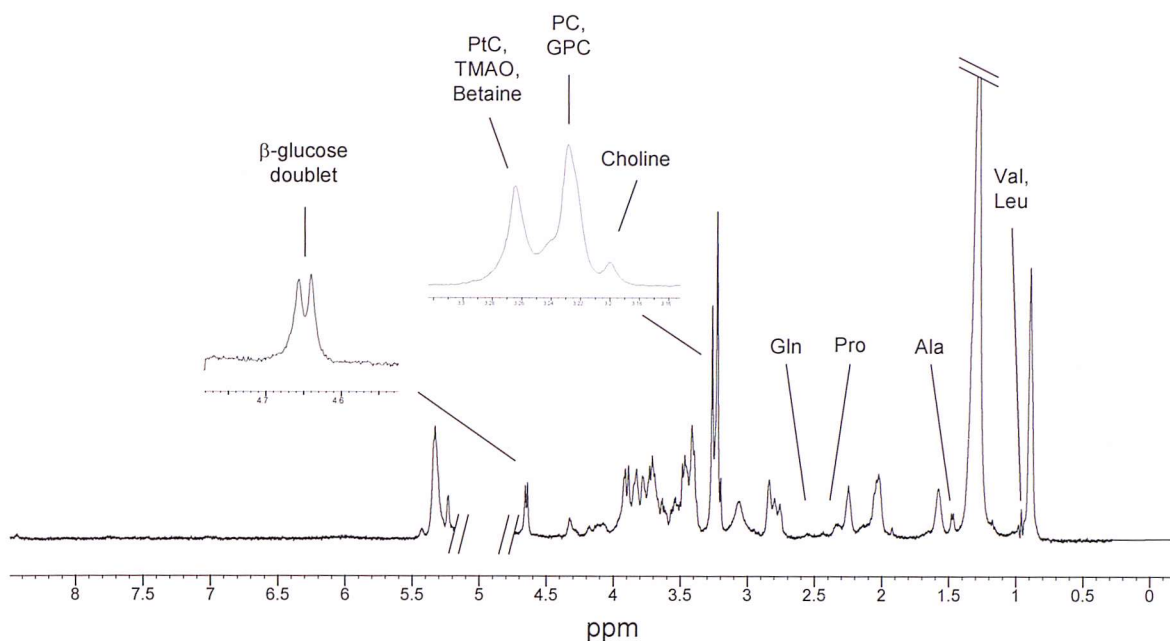


Figure 2-4. Spin-echo pulse sequence with water suppression.

A CPMG spectrum, TE 40ms, shows similar resonances to the lipid spectrum, but the broad components are removed. There is clear resolution of the choline region (see inset) including choline itself, phosphocholine (PC), glycerophosphocholine (GPC), phosphatidylcholine (PtC) with contributions from trimethylamine-*N*-oxide (TMAO) and betaine. The β -glucose doublet, used for optimisation experiments is also enlarged (inset).

2.6.3.1.1 Number of scans (acquisitions)

MRS is a technique of inherently low sensitivity. In order to increase the signal-to-noise ratio, the number of data acquisitions (scans) is increased. As the number of scans (N) increases, the signal increases by a factor of N and the noise, being random, by $\sqrt{N}$: accordingly, a doubling of scans leads to an increase in SNR of $\sqrt{2}$. Smaller samples or samples of low concentration provide less signal, so require more scans to derive a satisfactory SNR. Sequences that suppress components of the signal, such as spin-echo sequences, also require more scans. The number of scans performed is limited by the conflicting pressure of the time taken to perform the investigation and the degradation of the sample that might be expected to occur over that timescale.

2.6.3.1.2 *Repetition time and acquisition time*

T_1 exceeds T_2 for a given moiety in a specified physico-chemical environment. Biological tissue, being chemically and physically complex, contains multiple T_1 and T_2 components.

The repetition time (TR, in seconds) is the time from the start of one RF pulse to the start of the next RF pulse. TR consists of the acquisition time and the relaxation delay. If the TR is short relative to some or all of the T_1 components, there is insufficient time for the nuclei to relax between RF pulses, leading to incomplete signal generation; the signal is said to be “saturated”.

During the acquisition time, nuclei undergo T_2 relaxation, and the acquisition time needs to be selected to allow measurement of short and long T_2 components. It is defined by the spectral sweep width and the number of data points collected.

In order to ensure that the spectra were fully relaxed with all components represented, a TR of 10s was chosen for pulse and collect sequences without water suppression and 5s for pulse and collect sequences with water presaturation, and CPMG sequences. The longer TR was chosen for the spectra including water, as long water T_1 components had been observed (IJ Cox, personal communication). By using 32k datapoints and a spectral width of 15kHz, an acquisition time of 2.2s allowed complete detection of resonances with linewidths of less than 1Hz.

2.6.3.1.3 *Echo time*

Spin-echo sequences, such as CPMG, cause a refocussing of the transverse relaxation decay by reversing the dephasing of nuclei by application of a 180° inversion pulse after a time interval, the echo time (TE). By changing the TE, the signal from spectral components of a specified range of relaxation times may be selectively detected.

2.6.3.1.4 *Pulse angle*

The pulse angle is the angle at which the RF pulse is applied, relative to $\mathbf{B}_0$. A pulse angle of 90° leads to maximal deviation of dipoles from $\mathbf{B}_0$ and, accordingly, to maximal signal production.

Where samples are large or expected to generate a large signal, the signal may exceed the spectrometer capacity, leading to truncation of the signal. In this situation, the pulse angle may be reduced to reduce the absolute signal generated. Signal amplitude is proportional to sine of the pulse angle ($\sin\theta$).

2.6.3.1.5 *Spectral width*

The spectral width (or sweep width) is the frequency range over which the signal is collected and was set at 15kHz to prevent the “spinning-sideband” artefact from overlapping with the metabolite peak of interest (Mahon *et al.*, 2004a). The digital resolution of the spectrum is the spectral width divided by the number of datapoints.

2.6.3.1.6 *Receiver gain*

The receiver gain magnifies the FID signal induced in the receiver coil. Therefore, absolute signal intensity may be modified by the pulse angle, the receiver gain, or both.

2.6.3.2 *Choice of parameters for in vitro NMR spectroscopy studies*

Initial murine studies were carried out using sequences developed for the analysis of human oesophageal and cervical tissue performed using the same hardware (Dave *et al.*, 2004; Mahon *et al.*, 2004b) and are shown in **Table 2-2**. The pulse angle was reduced in pulse and collect spectra with and without water suppression to prevent the signal exceeding the spectrometer capacity when large or lipid-rich samples were used. The time of acquisition and number of scans acquired were limited to minimise the effects of sample degradation.

Table 2-2. NMR parameters chosen for initial murine experiments

Sequence	Scans	Receiver gain	Pulse angle (°)	Relaxation delay (s)	Echo time (ms)	X points (k)	Spectral width (kHz)	Time (min)
Pulse and collect	16	15	9	10		32	15	5
Water presaturation	64	15	45	5		32	15	8
CPMG	64	15	90	5	40	32	15	9

Abbreviations: X points, number of datapoints along the x axis; CPMG, Carr-Purcell-Meiboom-Gill sequence.

2.6.4 Spectral processing functions

Once MR data had been acquired, FIDs were imported from the JEOL Eclipse 500+ MR spectrometer to the processing software. ¹H MAS NMR spectra were managed using the KnowItAll® Informatics System v7.8 (Bio-Rad, Philadelphia, USA). The following processes were applied:

2.6.4.1 Exponential windows function (apodization)

The exponential windows function adds a weighting to the FID, which amplifies the signal more than the noise, thus increasing SNR.

2.6.4.2 Zero filling

Zero filling increases digital resolution by adding null data to the end of the FID. The level of zero filling is selected automatically on the basis of the number of experimentally collected datapoints. Zero filling has the effect of smoothing data and increasing resolution.

2.6.4.3 DC offset

The NMR signal can be shifted from the baseline on account of instrumental errors. This artefact is corrected during processing.

2.6.4.4 *Phasing*

The received NMR signal, the FID, takes the form of sine waves. The value of the waveform on the y axis at a given time may be positive, negative or anywhere in between. This depends on the phase of the wave, which depends on the time at which measurement starts and the direction from which the signal is measured. Following the delivery of an RF pulse, the receiver may be overloaded initially (the deadtime). Different components of the spectrum evolve during the deadtime, so may be out of phase when the receiver starts to detect the signal. If these components are out of phase, signal may be lost. Therefore, phase correction mathematically corrects the phase of the components at the start of data acquisition to ensure a symmetrical, positive lineshape upon Fourier transformation.

All data processed in Knowitall[®] were subject to automatic GoodLook[™] phasing, systematically optimising the phase parameters to maximise the peak integral above the baseline. Manual phasing was performed if automatic phasing did not appear optimal.

2.6.4.5 *Baseline correction*

Baseline correction corrects severe baseline bias which causes a systematic error to the y-values of part of the spectrum. Using Knowitall[®], an automatic spline algorithm is applied, which fits a number of polynomial curves to the spectral baseline, excluding spectral peaks.

2.6.4.6 *Referencing*

The chemical shift is defined as a difference in frequency between the peak of interest and the reference peak. A reference may be internal or external to the sample. An ideal reference is a well-defined peak, which is clearly identifiable in all spectra in a dataset, the position of which is independent of biochemical or physical changes, such as differences in pH and temperature. In plasma and serum, the lactate doublet at $\delta 1.32\text{ppm}$ is often used as a reference (Kirschenlohr *et al.*, 2006), but this peak is often not visible in liver tissue samples, where it is often obscured by the large lipid resonance centered at $\delta 1.30\text{ppm}$. In murine fatty liver experiments, where the

spectra were dominated by lipid resonances, the reference peak used was that of the methyl lipid proton at $\delta 0.9$ ppm.

2.6.4.7 *Peak assignment*

2.6.4.7.1 *Analysis of samples of known composition*

The molecular structure of organic compounds may be inferred from the relative size and position of resonances, which correspond to the relative abundance of nuclei and the bonds or coupling between them. An external reference compound may be added, so that the chemical shift may be measured. Reference databases of spectral signatures have been established (Wishart *et al.*, 2007).

2.6.4.7.2 *Correlation of resonances in complex biological samples*

In complex samples, NMR spectral signatures overlap and the relative concentrations of constituents may not be known. Resonances may be assigned by “spiking” the sample with substances of a known chemical shift pattern. Two-dimensional spectroscopy techniques, such as COSY (correlation spectroscopy) and HMQC (heteronuclear multiple quantum correlation) allow nuclear interactions to be studied. COSY combines spectra to illustrate coupling between proton pairs. Coupling may occur over a distance of two to three bonds, so complex structures may be assembled. HMQC plots spectra from different nuclei on each axis, allowing correlation between the assignments of resonances from spectra of one nucleus to the other. Combining this approach with information from pure sample databases allows the assignment of resonances in previously unstudied sample types. An early study of intact rat liver tissue by MAS NMR spectroscopy used total correlation spectroscopy (TOCSY) and HMQC to assign the resonances obtained (Bollard *et al.*, 2000). Subsequent studies by independent groups have examined human liver tissue using similar techniques to publish databases of metabolites to be found in intact liver tissue (Duarte *et al.*, 2005; Martinez-Granados *et al.*, 2006).

2.6.4.7.3 *Assignment on the basis of the published literature*

The publication of databases of the relevant resonances for the tissue to be studied allows assignment of resonances in subsequent studies, although care should be taken not to extrapolate these databases beyond the sample type used. For the studies presented in this thesis, a database of the spectral assignments from three independent studies of intact liver tissue in rodent and humans was compiled for the assignment of resonances (Bollard *et al.*, 2000; Duarte *et al.*, 2005; Martinez-Granados *et al.*, 2006). The published chemical shifts were largely in agreement between studies, increasing confidence in the results. Where there was ambiguity or conflicting information, further references were sought as specified.

2.6.5 Integration methods

2.6.5.1 *Signal to noise and linewidth measurement*

The signal to noise ratio (SNR) was calculated by dividing the height of the β -glucose peak at $\delta 4.65$ ppm by the width of the noise band at $\delta 4.5$ ppm (peak to trough). The line width was defined as the breadth of the β -glucose doublet at half peak height. If two peaks were discernable at the β -glucose resonance, a doublet was considered to be visible. An optimal spectrum was considered to have a high SNR, a narrow line width, and to allow clear subjective assessment of a β -glucose doublet where present. The β -glucose resonance was chosen as it is easily identifiable, stands alone without interference or overlap from neighbouring peaks and consists of a doublet, which allows for a subjective interpretation of spectral resolution.

2.6.5.2 *Integration of pre-defined regions*

Quantification of metabolites was performed by integration of resonances, the area under the curve being proportional to the number of nuclei contributing to that resonance. Regions for integration were defined by placement “bins” or “buckets” defining the extent of the baseline subtending the region of interest. Such a technique is ideal for the integration of narrow, well-defined and well-separated resonances, such as those obtained by solution-state work (Salek *et*

al., 2007). In such spectra, there is little overlap between resonances and the nadir between peaks falls to baseline. ^1H MAS NMR spectra from intact liver tissue approximate to that obtained from solution-state work (Rooney *et al.*, 2003). However, the presence of cell membranes and connective tissue, which are absent in plasma, leads to broader resonances. The water presaturation sequences of liver tissue are also dominated by the lipid resonances. Smaller narrow resonances from metabolites, such as free amino acids, may be superimposed over the “shoulder” of the lipid resonance, with the nadir between peaks not reaching baseline. For most analyses, the peaks of interest were assumed to be sufficiently separated for integration of a bin subtended by the curve to be considered a close surrogate of the true peak integral.

2.7 Results of calibration experiments

2.7.1 Physical parameters

A number of physical parameters affect the tissue and the resultant NMR spectra. These have been examined in turn to determine the optimum experimental design for subsequent ^1H MAS NMR studies.

Theoretically, the signal amplitude received from a sample is proportional to the number of nuclei in the sample. Accordingly, a greater signal is expected from a sample of similar chemical constituents with a higher mass when all other parameters are kept constant. However, there is the potential for physical properties of biological tissue to interfere with quantification of spectral resonances.

If a tissue sample is held in a strong magnetic field, a magnetic field gradient will vary over the sample according to the shape of the sample, tissue architecture and chemical constituents. Accordingly, nuclei in the same stereo-chemical environment can experience a different strength magnetic field, so will resonate at a different frequency and therefore at a different chemical shift. This leads to broadening of the peak in the frequency domain and reduction in spectral resolution. The process of shimming adjusts the applied magnetic field, reducing magnetic field strength gradients over the sample in order to minimise line broadening.

In order to investigate the effect of sample mass, sealing cell volume and sealing cell shape on spectral quality, normal murine liver from 8 week-old *cd1a* mice was analysed using differing sample masses, cell volumes and cell cavity shapes. Pulse and collect spectra with water presaturation were used with NMR parameters kept constant. Shimming was optimised manually by a single operator.

2.7.2 Sample mass

To assess the effect of sample size on SNR and linewidth, NMR spectra from 11 samples of normal murine liver tissue of mass varying from 2-20mg, placed in 20 μ L sealing cells, were analysed retrospectively. On examination of the pulse and collect with water presaturation sequences, there was close correlation between sample mass and SNR, Spearman rho $r=0.92$, $p<0.0001$. The linewidth correlated more weakly with sample mass, Spearman rho $r=0.63$, $p=0.04$. The increase in linewidth seen in larger samples may have resulted from greater magnetic field inhomogeneity found in larger samples, but the effect of linewidth is accounted for in the calculation of SNR. These relationships are displayed in **Figure 2-5**. Accordingly, larger samples were considered preferable to smaller samples where sample size was not the limiting factor.

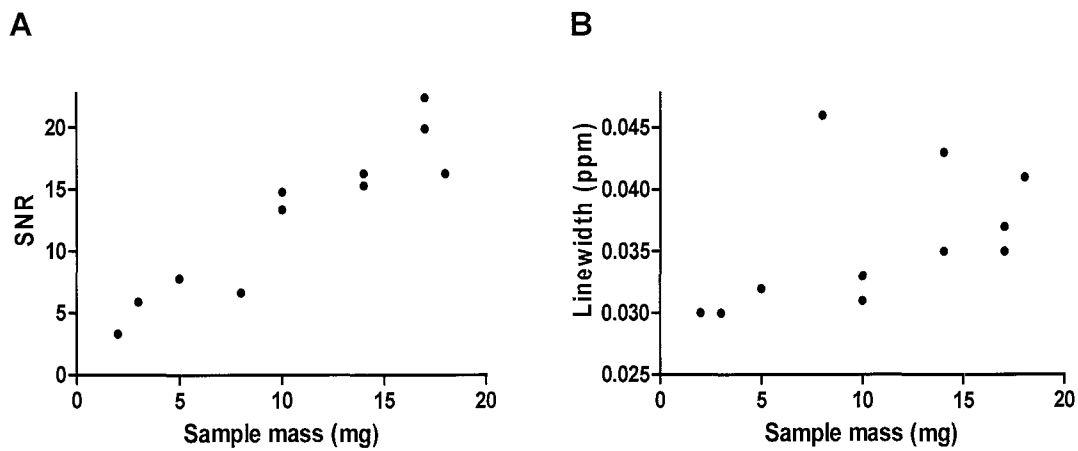


Figure 2-5. Sample mass plotted against signal to noise ratio and linewidth.

A, signal-to-noise ratio (SNR) and B, linewidth.

2.7.3 Sealing cell volume

To assess the effect of chamber size on SNR, normal murine liver samples of matched mass and provenance were placed in 20 μ L cylindrical cells ($n=3$) and 4 μ L spherical cells ($n=3$). SNR and linewidth were calculated as described previously. The mean $\pm$ SEM sample mass was 3.3 ± 0.8 mg in the 20 μ L cells and 3.0 ± 0.06 mg in the 4 μ L cells (unpaired t-test, $p=0.73$). The mean $\pm$

SEM SNR was 17.1 ± 3.90 and 32.5 ± 5.61 respectively ($p=0.13$). The mean $\pm$ SEM linewidth was 0.031 ± 0.001 ppm versus 0.032 ± 0.002 ppm respectively, ($p=0.46$). In this small study, there was a suggestion that the SNR was greater for a given sample mass when analysed in the small 4 μ L spherical cavity, although the linewidth was unchanged.

The receiver coil was focused to the centre of the sealing cell. Accordingly, signal from the centre was received maximally. Where a small sample was placed in a large cell, the sample was distributed evenly around the periphery of the cell, leaving little tissue in the centre. A similar sample in a small cell will remain centrally distributed, yielding a higher total signal. A centrally placed sample might also be expected to provide a narrower linewidth. This was not found in practice using tissue samples and fine resolution was, in fact, more difficult to achieve. This may be explained by the larger shearing forces experienced by a small sample in a large cell compared to a similar sample in cell with a small spherical chamber. Accordingly, such a sample will experience greater structural disruption, leading to enhanced molecular mobility and, therefore, decreased linewidth.

As a result of these experiments, where sample mass was unlimited, large samples (>10mg) were inserted into 20 μ L sealing cells. Where sample size was limited, the smallest cell able to contain the whole sample was used to reduce the shearing forces experienced, increase the magnetic field homogeneity and yield the maximal SNR.

2.7.4 Temperature of data acquisition

In order to assess how sample temperature affects the observed spectrum, three samples of normal murine liver tissue were analysed using pulse and collect sequences with water presaturation at the following temperatures: 0°C, 4°C, 25°C and 37°C before returning to 0°C. These temperatures were selected to represent temperatures used in previously published studies and mammalian body temperature (Bollard *et al.*, 2000; Duarte *et al.*, 2005; Martinez-Granados *et al.*, 2006). The SNR and linewidth were calculated as described above. The sample

temperature also determines the chemical shift of water (ppm) according to **Equation 2-B** (Gottlieb *et al.*, 1997):

Equation 2-B

$$\delta = 5.051 - 0.0111(T)$$

where δ is the chemical shift in ppm and T is the sample temperature in °C. As a result, at 0°C, water is at $\delta 5.05$ ppm and at 37°C, the water peak is at $\delta 4.64$ ppm.

The SNR increased with successively increasing temperatures but did not increase further when the temperature returned to 0°C, with a decrease in SNR (non-significant). The linewidth decreased with successively increasing temperatures but did not decrease further when the temperature returned to 0°C, with a small increase in linewidth (non-significant) (**Figure 2-6**). This shows that, at higher temperatures, there was an increased SNR and a narrower linewidth. However, as the measurements at 0°C separated by time were not equal, an irreversible change may also have occurred, which may represent sample degradation. Although a higher acquisition temperature appeared to increase spectral quality (as assessed by improved SNR and linewidth), the effect of sample degradation with time must be considered.

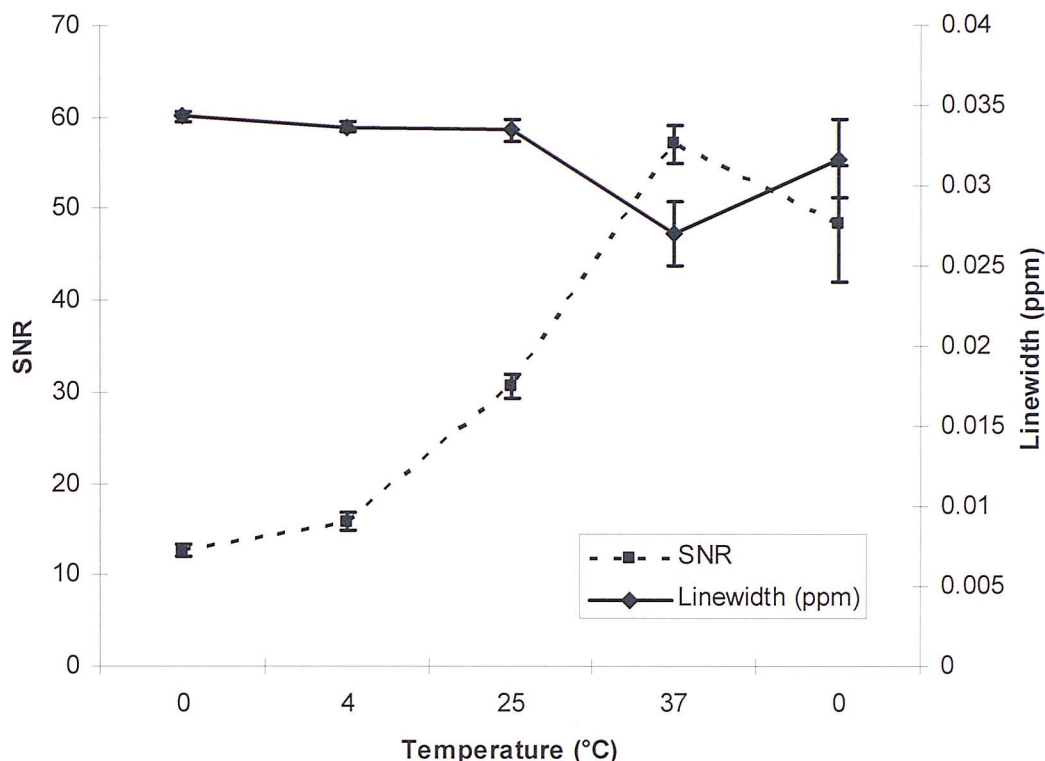


Figure 2-6. Change in signal-to-noise ratio and linewidth with temperature.

The mean ± SEM SNR and linewidth of the β -glucose resonance, $n=3$, plotted against temperature of acquisition and altered sequentially from left to right.

2.7.5 Duration of acquisition

It has been shown previously that sample spinning leads to spectral changes with time when compared to a matched sample kept motionless at the same temperature, particularly within the glucose and glycogen regions (Martinez-Granados *et al.*, 2006).

On the basis of these data, it was hypothesised that sample spinning leads to application of mechanical shearing forces to the sample, with disruption of tissue and cellular architecture. Such disruption decreases magnetic inhomogeneities and increases fluidity within the sample, causing an increase in T_2 relaxation times and consequent reduction in linewidth and increase in subjective spectral resolution.

The rate of change of the metabolic profile was examined by comparing the spectra from three samples spun at 4°C and three samples spun at 25°C, over a 4hr period. Acquisition temperatures were selected from previous studies (**Table 2-1**). The samples of histologically normal murine liver were placed in 20μL sealing cells as described above. The mean ± SEM weight was 15.4 ± 1.5 mg for the samples at 4°C and 15.2 ± 0.7 mg for the samples at 25°C. Fully relaxed ^1H MAS NMR spectra with water presaturation were acquired every 20min for 4hr.

The lipid -CH₂ and total choline resonances were integrated as representative of high molecular weight, lipid resonances and membrane metabolites. The alanine doublet at $\delta 1.48$ ppm and the β -glucose doublet at $\delta 4.65$ ppm were integrated as representative of the low molecular weight metabolites. These resonances were chosen as they were well-defined and did not overlap substantially with other peaks. The SNR and line width were calculated as described above.

The mean signal for the lipid -CH₂ resonance was significantly reduced by 1hr from baseline and remained so at 4hr at both 4°C and 25°C. The mean signal for alanine was significantly increased at 4hr at 25°C but not 4°C and in neither group at 1hr. The mean signal for β -glucose was significantly increased at both 1hr and 4hr at 25°C but not at 4°C (**Table 2-3**).

The mean ± SEM signal intensity as a percentage of each of the measured resonances at 4°C and 25°C with time is represented in **Figure 2-7**. There was a significantly greater change at 25°C compared to 4°C in both alanine and β -glucose resonances at 4hr. Quantitative comparison of signal intensity with time is displayed in **Table 2-3**. Significant changes in signal intensity over time are seen in the methylene lipid group at 4°C and in the methylene lipid, alanine and β -glucose resonances at 25°C.

Table 2-3. Change in integrals of resonances with time at 4°C and 25°C.

	4°C			25°C		
	0 hr	1 hr	4 hr	0 hr	1 hr	4 hr
Lipid CH₂	10.64 (1.72)	9.99* (1.92)	8.85* (2.01)	8.98 (1.76)	7.95** (1.85)	6.70** (1.42)
Alanine	0.48 (0.16)	0.46 (0.15)	0.51 (0.13)	0.42 (0.06)	0.52 (0.07)	0.81* (0.16)
Choline	2.19 (0.46)	2.22 (0.42)	2.19 (0.34)	2.51 (0.38)	2.64 (0.34)	2.89 (0.23)
β-glucose	0.57 (0.08)	0.60 (0.06)	0.64 (0.06)	0.59 (0.10)	0.71* (0.13)	0.82** (0.12)

*Mean integrals for spectral peaks with standard deviation (s.d.) in arbitrary units showing change in values with time at 4°C and 25°C. Significant changes at the $p < 0.05$ level, compared to baseline (start), are denoted by *, while changes significant at the $p < 0.01$ level, compared to baseline are denoted by **.*

These data demonstrate that changes are observable in ¹H MAS NMR spectra of normal liver samples over time. The changes are more marked and more extensive when the spectra are acquired at 25°C as opposed to at 4°C. **Figure 2-7** shows that there is a linear relationship between these changes and time, so the effects of sample degradation must be considered when choosing the number of data acquisitions to use for a sequence.

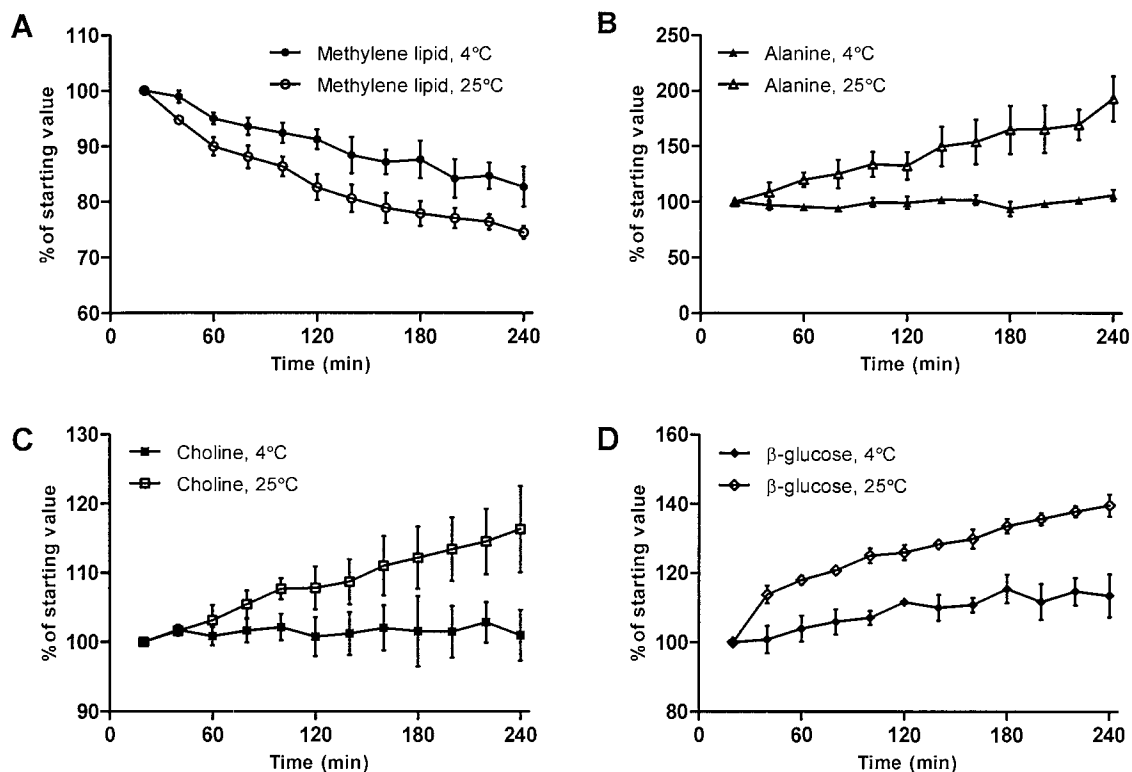


Figure 2-7. Change in signal over time for four metabolite resonances.

A, methylene lipid; B, alanine; C, choline and D, β-glucose at 4°C and 25°C. Points demonstrate mean $\pm$ SEM signal as a percentage of the starting value.

2.7.6 Spin speed

Spinning of the sample should be at a rate sufficient to remove the spinning sideband artefact from the region of interest (0-10ppm). The distance of the spinning sideband from the region of interest may be calculated by **Equation 2-C**:

Equation 2-C.

$$\text{Distance of spinning sideband (ppm)} = \text{Spin speed (Hz)} \times 10^6 / \text{Operating frequency (MHz)}$$

The operating frequency is 500MHz, so spinning at 2000Hz gives sidebands at ± 4 ppm and at 4000Hz gives sidebands at ± 8 ppm from the parent peak. The greatest sidebands are produced by water at $\delta 4.8$ ppm and lipid $-\text{CH}_2$ at $\delta 1.3$ ppm and the useful spectrum is found between $\delta 0$ ppm and $\delta 8$ ppm. Therefore, in order that sidebands do not interfere with the spectrum

produced, they should be more than 8ppm from the parent peak, requiring a minimum spin speed of 3500Hz. ^1H MAS NMR spectroscopy studies of intact liver tissue have used a spin speed of 4000Hz when the operating frequency is between 400 and 500MHz (Bollard *et al.*, 2000; Duarte *et al.*, 2005; Martinez-Granados *et al.*, 2006).

High frequency spinning causes the generation of shearing forces within the sample, leading to disruption of tissue structure and acceleration of sample degradation. This factor was shown to be additional to degradation caused by the sample existing at a defined temperature (Martinez-Granados *et al.*, 2006).

2.8 Discussion of NMR and physical parameters

2.8.1 NMR parameters

The choice of NMR parameters involves some compromise between competing phenomena. Thus, the aims of any studies should be considered when making these decisions. As the overall aims of the project were to assess the metabolic profiles of liver tissue in chronic liver disease and to use this information to inform the analysis and interpretation of MR spectra *in vivo*, the *total* spectral information was of interest. The use of fully-relaxed pulse and collect sequences, with and without water suppression, enabled the simultaneous assessment of aqueous and lipid components (**Figure 2-2** and **Figure 2-3**). These sequences also enabled ratios of metabolites to be assessed without the introduction of spectral-editing-related bias, caused by the relative suppression of some metabolites over others. For full-relaxation and maximal signal production, long TRs were chosen (**section 2.6.3.1.2**), which increased acquisition time, but enabled spectra with good SNR to be obtained with fewer transients or averages, thus reducing the acquisition time. Reduction of the acquisition time is also important to limit sample degradation. In contrast to previous studies, we have shown significant changes in resonances within 1hr at both 25°C and 4°C (Bollard *et al.*, 2000; Martinez-Granados *et al.*, 2006). The rate of degradation cannot be assumed to be equal from sample to sample or tissue to tissue, so, for the *in vitro* metabolic profiles to represent the metabolite ratios found *in vivo*, minimal degradation should be allowed to occur. This may, therefore, conflict with the need to obtain spectra with a maximal SNR.

However, for the assessment of certain spectral regions, spin-echo (CPMG) sequences were used to suppress macro-molecular components over low molecular weight metabolites. This enabled the examination of smaller signals, which might be obscured by large lipid resonances. However, the spin-echo sequences reduce signal from all parts of the spectrum, so more transients were required to obtain high SNRs. This takes more time and so leads to potential degradation. By choosing a longer echo time, macromolecular signals were suppressed more, but more transients were required to obtain an appropriate SNR.

Maximal signal was obtained from a pulse angle of 90°, but in large samples there is a risk of signal saturation, which is mitigated by reducing the receiver gain. If the receiver gain is not specified, the signal amplitudes cannot be compared in absolute terms between samples. However, the studies in **Chapter 3** were unaffected by saturation and spectral processing normalised the spectra to the total spectral integral, so absolute signal measurement was not required. For subsequent experiments (**Chapter 4**), 90° pulse angles were used.

As a result of these considerations, the NMR parameters stated in **Table 2-2**, were revised for the studies of human tissue in **Table 2-4**.

Table 2-4. NMR parameters optimised for subsequent experiments.

Sequence	Scans	Receiver gain	Pulse angle (°)	Relaxation delay	Echo time (ms)	X points (k)	Spectral width (kHz)	Time (min)
Pulse and collect	32	5	90	10		32	15	11
Water presaturation	128	15 (10-18)	90	5		32	15	16
CPMG	256	15 (10-18)	90	5	80	32	15	32

Abbreviations: X points, number of datapoints along the x axis; CPMG, Carr-Purcell-Meiboom-Gill sequence.

2.8.2 Physical parameters

A sample with a greater mass yields a higher signal-to-noise ratio for a given number of data acquisitions owing to the proportionately greater number of protons. However, differing amounts of a given metabolite in different samples will also affect the signal produced and therefore the SNR. The calibration experiments were carried out using normal 8 week old CD1a

mouse liver, so metabolite differences between samples is likely to be small. A large sample therefore increases the SNR.

A larger sample is likely to be more representative of the organ of interest, as demonstrated by studies of the diagnostic performance of human liver biopsies (Bedossa *et al.*, 2003). However, occupation of a larger volume, either through a larger sample or a larger sample size, leads to greater magnetic field inhomogeneities throughout the chamber. This is demonstrated by an increased linewidth seen in the larger samples and potentially decreased spectral resolution as represented by the ability to determine the presence of a β -glucose doublet subjectively. Therefore, a smaller chamber, which may necessitate a smaller sample, should yield a narrower linewidth. Accordingly, where tissue availability was not strictly limited, a sample size of 15-18mg within a 20 μ L sealing cell was considered optimal. Where tissue availability was limited, as when analysing samples from human liver biopsies, the sample was placed in the smallest sealing cell that would contain the whole sample.

2.9 Discussion of spectral processing

The choice of spectral processing functions, like that of NMR parameters, required compromise between functions, depending on the information required for a given analysis. Phasing and baseline correction should be consistent between samples, as differences in these functions may give rise to altered signal amplitudes, introducing further uncontrolled variables into the dataset. Therefore automatic functions were used where possible, with subjective assessment of each spectrum for quality control.

Methods for the integration of spectral resonance may also affect the data obtained. Use of a *metabonomic* approach to the analysis of ^1H MAS NMR spectra involves the division of the spectrum into numerous bins, which are integrated, as described in **section 2.6.5**. This approach is ideal where the resonances are numerous (hundreds), narrow ($\sim 1\text{Hz}$ linewidth) and well-separated, as each peak may be fully contained within a single bucket, with the nadirs on either side falling to baseline. In these circumstances, the approach is accurate and reproducible. However, where lineshapes are broad and overlapping, buckets are unable to assess the contribution of each of overlapping peaks to the composite whole. This is of particular importance *in vivo*, where spectral resolution is poor (5-15Hz linewidth). In these situations, individual curve-fitting programs may be used, where the contributing resonances are modelled using prior knowledge (as described in **section 5.5.2**). However, this approach is currently only suitable for the analysis of a few peaks (<10) on account of the software processing limits and is labour-intensive, making it unsuitable for the analysis of many spectra.

The spectra obtained by ^1H MAS NMR consist largely of narrow resonances, but, as both aqueous and lipid fractions are present, including cell-membrane components, composite or co-resonant peak are seen. Assumptions must therefore be made on the basis of the information required in the study. For example, where the lipid resonances are measured, as lipid peaks are dominant in the spectrum, the contribution from most co-resonant metabolites may be ignored. Where a smaller resonance is present on the shoulder of a larger peak, the contribution from the

larger peak must be assumed to be constant, or incorporated into the analysis as a ratio. Newer curve-fitting capabilities to metabonomic analysis software are in development (Dr C Peng, software engineer, BioRad, Philadelphia, USA, personal communication), but were unavailable for these studies.

2.10 Conclusions

The rationale for the use of ^1H MAS NMR spectroscopy to study intact liver tissue has been presented. NMR parameters and approaches to analysis have been discussed, where relevant to the experiments performed as part of this thesis. Previous studies using ^1H MAS NMR spectroscopy for the study of liver tissue in chronic liver diseases have been reviewed and differences in the technical approach have been highlighted. Specific physical parameters including optimal sample size, sealing cell volume, acquisition temperature and experimental duration have been assessed experimentally: a large sample in the smallest available chamber yields the optimal SNR and resolution; acquisition temperature was set at 4°C to reduce sample degradation and the duration of experiments did not exceed 1hr. Finally, approaches to spectral analysis have been discussed. As a result of these studies and on the basis of the issues discussed in this chapter, experimental conditions have been defined for subsequent studies within the thesis.

3 PHENOTYPING MURINE MODELS OF NAFLD

3.1 Abstract

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease, associated with the metabolic syndrome. Effective techniques are needed to investigate the potential of animal models of NAFLD. This study aimed to characterise murine models of NAFLD by metabolic profiling of intact liver tissue.

Mice of three strains (BALB/c, C3H and the novel mutant, Gena/263) were fed either a control or high fat diet. Biometric, biochemical and histological analysis demonstrated a spectrum of NAFLD from normal liver to steatohepatitis. Metabolic profiling of intact liver tissue, ^1H MAS NMR spectroscopy, showed an increase in total lipid-to-water ratio, a decrease in polyunsaturation indices and a decrease in total choline with increasing disease severity. Principal components analysis and partial least squares discriminant analysis showed separation of each model from its control and of each model from the total dataset. Class membership from the whole dataset was predicted with 100% accuracy in 6 of 8 models. Those models with steatosis discriminated from those with steatohepatitis with 100% accuracy.

Conclusion: the separation of histologically-defined steatohepatitis from simple steatosis is clinically important. Indices derived from ^1H MAS NMR spectroscopy studies may inform subsequent *in vivo* MR spectroscopy studies at lower field strengths.

3.2 Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of liver disease encompassing steatosis (fatty change), non-alcoholic steatohepatitis (NASH) and cirrhosis in the absence of alcohol abuse (Anstee and Goldin, 2006). Population studies show that NAFLD is strongly associated with insulin resistance, type II diabetes mellitus, hypertension, obesity and dyslipidaemia (Marchesini *et al.*, 1999; Powell *et al.*, 1990; Sanyal, 2002). The pathogenic mechanisms remain poorly understood and are difficult to study in humans (Koteish and Mae, 2002). While an estimated 10-24% of adults worldwide have a degree of hepatic steatosis (Angulo, 2002), only a small proportion of these go on to develop progressive disease, characterised histologically by steatohepatitis with fibrosis. In order to target therapy to the appropriate patients there is a need for accurate, well-validated, methods to analyse hepatic lipid content and metabolism.

In vivo, MRS has been used to assess the prevalence of NAFLD (Szczepaniak *et al.*, 2005); recently, more detailed information on lipid composition has been reported (Johnson *et al.*, 2008). *In vitro*, MRS has been applied to the study of liver tissue extracts in obese rats (Serkova *et al.*, 2006) and urine and plasma from mice with features of NAFLD (Dumas *et al.*, 2006; Fearnside *et al.*, 2008).

Much of the understanding of the pathogenesis of NALFD is based on research using animal models (Anstee and Goldin, 2006). No existing model exhibits the entire NAFLD phenotype. Many differ pathologically from human disease in all but superficial histological appearance. Thus, research is hampered by the lack of reliable models of progressive steatohepatitis that accurately reflect the human disease. Currently, the phenotyping of such models is reliant on physical observation, indirect biochemical assay and histopathology, all of which are time-consuming. In addition, subtle changes in metabolism and hepatic disease severity may not be detected using conventional techniques. Liver histology may be used to document overt features of NAFLD, such as the presence of hepatocellular lipid droplets, inflammation and fibrosis.

Histological assessment is semi-quantitative and involves a degree of subjectivity in interpretation. There is a requirement for robust techniques to phenotype rapidly and categorise the output of programs that develop new models of NAFLD. In particular, there is a need to identify animals which exhibit the characteristics of more advanced disease, including steatohepatitis.

3.3 Aims

The aims of this study were:

- a. To characterise murine models of NAFLD using ^1H MAS NMR spectroscopy of intact liver tissue.
- b. To assess the predictive value of multivariate models to determine the strain and diet for each mouse on the basis of ^1H MAS NMR spectroscopy differences.
- c. To compare NMR spectral differences between histologically characterised normal liver, steatosis and steatohepatitis.

3.4 Materials and methods

3.4.1 Animals

The study was approved by the local ethics committee and performed in accordance with the Animal (Scientific Procedures) Act 1986. Mice were housed under standard conditions (12hr light/dark cycle, temperature $21\pm 2^{\circ}\text{C}$, humidity $55\pm 10\%$) and provided with food and access to drinking water *ad libitum*. Two inbred strains of mice, known to differ in susceptibility to dietary induced NAFLD, C3H/HeH (sensitive) and BALB/cAnNCrI (resistant) were used in these studies (each group, $n = 6$) (Fearnside *et al.*, 2008). In addition, a recently described genetic model of NASH maintained on a C3H/HeH background, Gena/263 “Buttermouse”, was studied (each group, $n = 6-10$). The Gena/263 model was identified using a phenotype-driven n-ethyl-n-nitrosurea (ENU) mutagenesis screen for impaired glucose tolerance and histological/biochemical evidence of steatohepatitis (Matthews *et al.*, 2007). Male animals were randomised at age 6 weeks to receive either control diet (CD) or a nutritionally replete high fat diet (HFD). Control diet (SDS, Witham, UK) contained 4% fat by mass (11.5% of kcal) and the HFD (Research Diets Inc., New Brunswick, USA) contained 24% fat by mass (45% of kcal). Detailed dietary constituents are published elsewhere (Cobbold *et al.*, 2008a).

The animals remained on the allocated diet for the remainder of the study. Body weight was measured at age 6 weeks, 20 weeks and where applicable, 36 weeks. Glucose tolerance assessed using an intraperitoneal glucose tolerance test (IPGTT) at age 12 weeks. Mice were culled by exsanguination under terminal anaesthesia at one of two time points, aged 20 or 36 weeks.

The liver was excised and half was snap-frozen in liquid nitrogen prior to storage at -80°C , while the remainder was placed in 10% formalin. Formalin-fixed tissue was processed into paraffin wax and the sections were stained with haematoxylin & eosin, prior to histological examination by an experienced hepatopathologist, who was blinded to the phenotypic data,

using the well validated NASH Clinical Research Network semi-quantitative scoring system for cellular morphology, inflammation and fibrosis (Kleiner *et al.*, 2005).

3.4.2 NMR studies

Two samples were analysed from each liver (mean sample mass 15.3mg, sd 2.9mg). Freshly thawed liver tissue was placed into XC4 Kel-F sealing cells (Doty Scientific Inc., Columbia, South Carolina, USA).

3.4.2.1 ^1H NMR spectroscopy

^1H MAS NMR spectroscopy experiments were carried out using the equipment and methods described in **section 2.6**, and the parameters documented in **Table 2-2**. The pulse and collect sequence was used to derive a fully relaxed spectrum without suppression of the water resonance, the “total signal spectrum”. A pulse and collect with a water presaturation sequence provided water suppression, producing a spectrum dominated by lipid. A Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with water presaturation was employed to suppress macromolecular signal (mainly lipid) and emphasize lower molecular weight metabolite signals. In particular, this enabled the resolution and quantification of the resonances within the total choline region. 32K data points were acquired for each spectrum with a spectral width of 15kHz (Mahon *et al.*, 2004a). The overall time for data acquisition was 35 minutes.

3.4.3 Data analysis and interpretation

Spectra were processed using KnowItAll® Informatics System v7.8 (Bio-Rad, Philadelphia, USA). Free induction decays were zero-filled by a factor of 2 and multiplied by an exponential windows function with a 1Hz line-broadening function prior to Fourier transformation. Spectral resonances were assigned according to the published literature (Bollard *et al.*, 2000; Duarte *et al.*, 2005; Martinez-Granados *et al.*, 2006; Rooney *et al.*, 2003). Analysis was divided into hypothesis-generating multivariate analysis and biologically relevant conventional analysis of specific metabolites.

The spectral range of $\delta 0$ -10.0ppm was analysed, including the water region for the pulse and collect sequences and excluding the region containing the residual water resonance ($\delta 4.8$ -5.2ppm) in the sequences with water-presaturation. Spectra were scaled to the sum of the total spectral integral (water included for sequences *without* water suppression and excluded for those *with* water presaturation) to enable relative quantitative analysis of metabolites between samples.

3.4.4 Multivariate data analysis

Multivariate data-reduction techniques were applied in order to use information from the whole spectrum, and to determine which peaks contributed to the metabolic variation between individual samples and between groups of samples. NMR spectra acquired using the pulse and collect sequence with water presaturation were divided into small regions (“buckets”) of 0.04 ± 0.02 ppm, representing specific metabolite functional groups, placed using an “intelligent bucketing” algorithm in the Knowitall[®] software. The integral of each bucket was then quantified and expressed as a proportion of the total spectral integral, as discussed in detail in **section 2.6.5**.

Unsupervised multivariate factor analysis was performed using principal components analysis (PCA) to assess clustering and the presence of outliers. PCA is an unbiased technique, which is independent of any classifying information (such as strain) and the output summarises the metabolite variation within the dataset as a whole. Each principal component represents a combination of peaks, representing metabolites, expressed as a vector. The principal components are combined orthogonally, avoiding duplication or redundancy of information. Scores plots show the position of the samples in the newly-defined multidimensional space, where the axes represent each orthogonal principal component. Examination of the loadings plots allows identification of those metabolites which predominantly account for variability within the dataset.

Supervised analysis was performed using partial least squares discriminant analysis (PLS-DA) to investigate factors associated with class membership, with the Pirouette v4.0 software package (Infometrics, Washington, USA). Here, the classifying information is included in the analysis, so the output summarises the MR spectral (metabolic) differences between the groups. Orthogonal signal correction (OSC) is a filtering technique applied to PLS-DA analyses to remove variation in the spectra, which is unrelated to the defined conditions (Gavaghan *et al.*, 2002). To determine the predictive power of the model, cross-validation was performed by leaving out each sample in turn (a leave-one-out algorithm), and a model was constructed from the remaining samples to predict the class membership of the excluded sample. Such an algorithm makes best use of a small dataset, where a separate validation dataset is not possible. The Q^2 value indicates the validity of the discrimination, with values >0.05 considered significant (Jones *et al.*, 2005; Westerhuis *et al.*, 2008).

3.4.5 Examination of spectral resonances

In order to examine the specific differences between metabolite ratios of prior interest and those identified in the loadings plots, peak integrals (proportional to the total quantity of the metabolite in the sample) were measured. The lipid:water ratio was derived from the pulse and collect spectra without water suppression, both as the sum of the integrals of all the fatty acid proton resonances and as the methylene proton:water peak expressed as a percentage. The polyunsaturated to monounsaturated fatty acid ratio index (PUFA:MUFA index) was derived from the pulse and collect spectra with water suppression, and comprised the resonance at $\delta 2.80\text{ppm}$ (defined as the diallylic bond protons found in polyunsaturated fatty acids and denoted as “PUFA”) divided by the resonance at $\delta 5.33\text{ppm}$ ($-\text{CH}=\text{CH}-$, or n -ene functional group, found in mono- and poly-unsaturated fatty acids, denoted here as “MUFA”). The polyunsaturation index (PUI) was defined as the diallylic resonance divided by the sum of the allylic, diallylic, methyl and methylene resonances, expressed as a percentage (Johnson *et al.*, 2008). Total choline (tCho) was derived from the pulse and collect spectra with water

suppression and comprised the summed integral of the choline region resonances from δ 3.18- δ 3.28ppm, expressed as a percentage of the total spectral integral. The tCho region was further analysed using the CPMG data. Specifically, the resonance at δ 3.26ppm, assigned as phosphatidylcholine (PtC) including contributions from betaine and trimethylamine-*N*-oxide (TMAO) and denoted as “PtC”, was compared to the tCho levels. The correct assignment of lipid and choline resonances was verified by comparison of spectra from lean and steatotic murine liver to an oil sample of known composition (**Figure 3-1**).

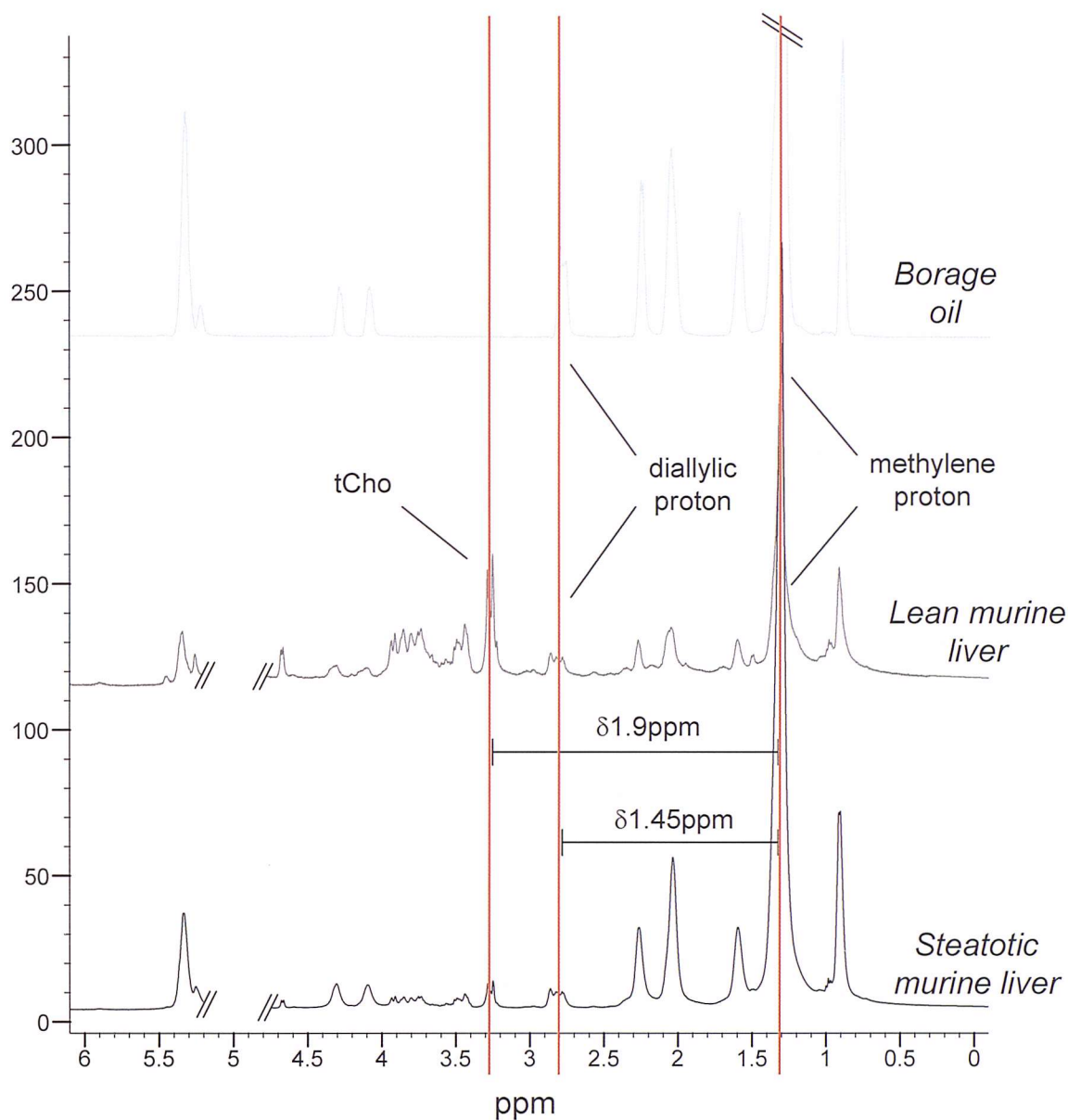


Figure 3-1. Alignment of MR spectral lipid resonances from oil and intact liver tissue.

High resolution ^1H MAS MR pulse and collect spectra with water suppression from lean and steatotic murine liver are aligned with a spectrum from a sample of borage oil acquired using the same parameters and referenced to the methylene ($-\text{CH}_2$) resonance at $\delta 1.3\text{ppm}$. Vertical red lines highlight the position of diallylic “PUFA” resonance at $\delta 2.7\text{--}2.9\text{ppm}$ and the choline resonances (total choline, tCho) found at $\delta 3.2\text{--}3.35\text{ppm}$ relative to the methylene resonance.

3.4.6 Conventional statistics

Significant differences in phenotypic data were sought by ANOVA testing with post-hoc Bonferroni analysis. Significant differences in MR spectral data were identified by the Kruskal-Wallis test (more than two groups) or the Mann-Whitney U test (pairwise comparisons) as appropriate (SPSS v14.0, SPSS Inc. Chicago, USA). Statistical advice was obtained from a biomedical statistician (Dr Bernard North, Statistical Advisory Service, Imperial College London).

3.5 Results

3.5.1 Phenotypic data

3.5.1.1 *Body weight*

At baseline there was no significant difference in body mass between the inbred strains. However, as shown in **Table 3-1**, by 20 weeks there were significant differences in body mass both between the inbred strains and the dietary groups (ANOVA, $p < 0.001$). Control diet (CD)-fed C3H mice gained weight more rapidly than CD-fed BALB/c animals ($35.32 \pm 2.57\text{g}$ versus $26.49 \pm 0.87\text{g}$; t-test, $p = 0.006$) and HFD-fed C3H animals gained the greatest weight, being significantly heavier than either CD-fed littermates ($44.72 \pm 0.34\text{g}$ versus $35.3 \pm 2.57\text{g}$; $p = 0.003$) or HFD fed BALB/c mice ($27.52 \pm 0.70\text{g}$; $p < 0.001$). Indeed HFD-fed BALB/c gained little weight and were not significantly heavier than CD-fed BALB/c mice. These differences increased during the course of the study with HFD-fed C3H animals being significantly heavier than all other groups at culling ($55.24 \pm 0.80\text{g}$ versus C3H-CD $43.31 \pm 2.58\text{g}$; $p = 0.001$; BALB/c-HFD $35.29 \pm 1.11\text{g}$; $p < 0.001$). Whilst HFD-fed BALB/c mice began to increase in weight compared with control this did not reach statistical significance. Throughout the study, the weight of mutant Gena/263 mice was not significantly different to wild-type C3H mice fed the equivalent diet.

Table 3-1. Phenotype of mice and effects of the high fat diet.

Strain	Diet	Mean Body Weight /g (SEM) [†]			Mean IPGTT at 12 Weeks /mmol/L (SEM)			Median NAS Hist. Score*			
		6 Weeks	20 Weeks	36 Weeks	T=0	T=60	T=120	St.	Fib.	Inf.	Ball.
C3H	Control	25.38 (0.59)	35.32 (2.58)	43.31 (2.58)	6.83 (0.22)	15.38 (1.94)	10.07 (0.41)	0	0	0	0
	HFD	25.24 (0.23)	44.72 (0.34)	55.24 (0.80)	10.42 (0.53)	21.26 (1.17)	11.86 (0.96)	2	0	1	1
BALB/c	Control	24.29 (0.83)	26.49 (0.87)	31.40 (1.53)	4.47 (0.99)	16.13 (2.48)	7.66 (1.52)	0	0	0	0
	HFD	23.25 (0.89)	27.52 (0.70)	35.29 (1.11)	3.58 (0.20)	11.34 (2.87)	7.30 (1.80)	0	0	0	0
		6 Weeks	20 Weeks	36 Weeks	Mean IPGTT at 12 Weeks /mmol/L (SEM)			Median NAS Hist. Score*			
					T=0	T=60	T=120	St.	Fib.	Inf.	Ball.
Gena/263	Control	24.56 (1.2) [‡]	41.60 (2.06)	-	10.84 (1.81)	26.66 (2.13)	22.43 (1.27)	1	0	0	2
	HFD	25.38 (0.46)	45.50 (0.64)	-	9.31 (0.95)	28.65 (1.42)	20.68 (0.53)	3	0	0	1
Non-Mut	Control	27.89 (0.5) [‡]	42.40 (2.57)	-	5.22 (1.93)	11.67 (0.29)	8.15 (1.14)	0	0	0	0
	HFD	26.58 (0.33)	44.76 (0.79)	-	8.42 (0.65)	23.43 (0.86)	13.30 (0.36)	2	0	0	1

Phenotype of mice and effects of the high fat diet (HFD) on body mass, glucose tolerance and histological scoring are tabulated. [†] Weight recorded at start of experiment (age 6 weeks), mid-experiment and at cull point. BALB/c and C3H were culled at 36 weeks, Gena/263 mice (and non-mutant, C3H controls) were culled at 20 weeks. [‡] 6 week control diet data from separate cohort of animals to those used in MRS study, shown for illustrative purposes. * NAS Score: St, Steatosis (0-3); Fib, Fibrosis (0-4); Inf, Inflammation (0-3); Ball, Ballooning hepatocyte degeneration (0-2).

3.5.1.2 Glucose tolerance

ANOVA analysis identified significant differences in blood glucose levels at all IPGTT time points (Table 3-1). HFD-fed C3H mice had significantly higher mean t=120 minute blood glucose concentrations than BALB/c mice fed HFD (11.86±0.96 versus 7.30±1.80 mmol/l, t-test, p<0.001). A trend towards impaired glucose tolerance was observed between HFD and CD-fed C3H animals but this did not reach statistical significance (11.86±0.96 versus

10.07±0.41 mmol/l). HFD fed Gena/263 mutant animals had significantly higher t=120 blood sugar levels than all other groups ($p<0.001$) including HFD-C3H mice (20.68±0.53 versus 11.86±0.96 mmol/l).

3.5.1.3 *Histology*

In keeping with the blunted weight gain and preservation of glucose tolerance in response to high fat diet, BALB/c mice were highly resistant to dietary induction of steatosis showing no histological evidence of steatosis in either CD or HFD groups at 36 weeks post-randomisation. In contrast, HFD fed C3H animals consistently exhibited a florid macro and micro-vesicular steatosis (median steatosis score 2/3) associated with ballooning hepatocyte degeneration (score 1/2) and a mild necroinflammatory infiltrate (score 1/3) (**Table 3-1**). Gena/263 mice exhibited mild spontaneous steatosis and a more moderate steatosis with some ballooning degeneration when fed HFD. A small but significant increase in serum ALT was observed between C3H dietary groups (HFD 47.60±4.41 versus CD 30.20±2.54 IU/L, $p=0.009$) and between HFD-fed C3H and BALB/c mice (47.60±4.41 versus 17.00±1.78 IU/L, $p=0.001$).

3.5.2 MR characterisation

A representative water-suppressed ^1H MAS NMR spectrum is shown in **Figure 3-2** and illustrates the specific, quantifiable lipid resonances, including the fatty acid functional groups. The choline region ($\delta 3.18$ - $\delta 3.28$ ppm) contains four clearly identifiable peaks representing choline ($\delta 3.20$ ppm), phosphocholine (PC) ($\delta 3.22$ ppm), glycerophosphocholine (GPC) ($\delta 3.23$ ppm) and PtC with contributions from TMAO and betaine at $\delta 3.26$ ppm. Glucose and glycogen resonances were also prominent. A number of amino acid resonances were also visible.

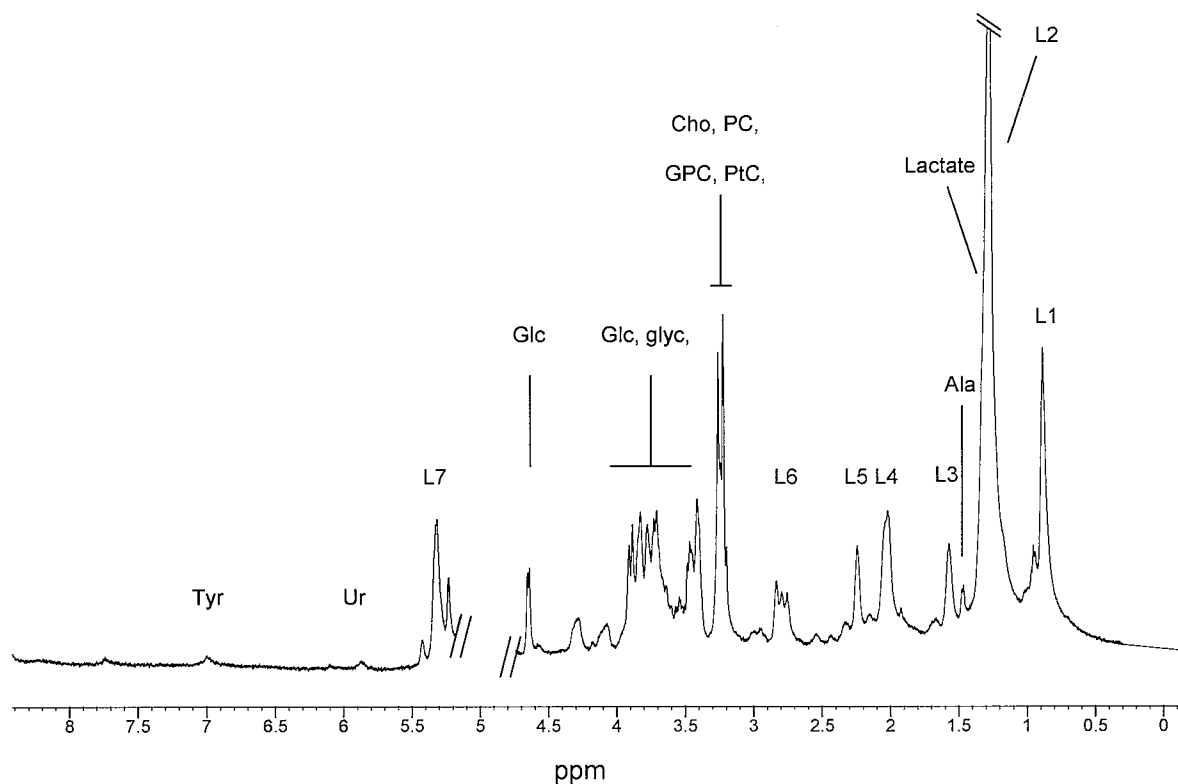


Figure 3-2. Water-suppressed ^1H MAS NMR spectrum from murine liver tissue.

Fully-relaxed pulse and collect ^1H MAS NMR spectrum, with water suppression from intact liver from a C3H mouse on control diet. The residual water resonance at $\delta 4.8\text{--}5.2$ was removed as indicated. Key: L1, lipid CH_3CH_2 (methyl); L2 lipid $(\text{CH}_2)_n$ (methylene); L3 lipid $\text{CH}_2\text{CH}_2\text{CO}$ (methylene to L5); L4 lipid $\text{CH}=\text{CHCH}_2\text{CH}_2$ (allylic); L5 lipid $\text{CH}_2\text{CH}_2\text{CO}$ (α -methylene to carboxyl); L6 lipid $\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}$ (diallylic- "PUFA"); L7 lipid $\text{CH}=\text{CH}$ (n -ene group- MUFA); Ala, alanine; Cho, choline; PC, phosphocholine; GPC, glycerophosphocholine; PtC, phosphatidylcholine; TMAO, trimethylamine- N -oxide; Glc, glucose; Glyc, glycogen; Urd, uridine; Tyr, tyrosine.

The sum of the integrals for all lipid peaks was compared to the integral of the methylene lipid peak in all samples, demonstrating strong correlation between measures (Spearman rho correlation, $r=0.998$, $p<0.0005$). Accordingly, the integral of the methylene proton resonance was considered a surrogate for total lipid when describing lipid quantities relative to water.

Principal components analysis scores plots demonstrated clustering and separation of each mouse model from its control (**Figure 3-3**). This included the separation of models on the basis of diet (CD or HFD), strain (BALB/c and C3H, both on CD and HFD) and also presence of the Gena263 mutation from the wildtype controls, both on CD and HFD (data not shown).

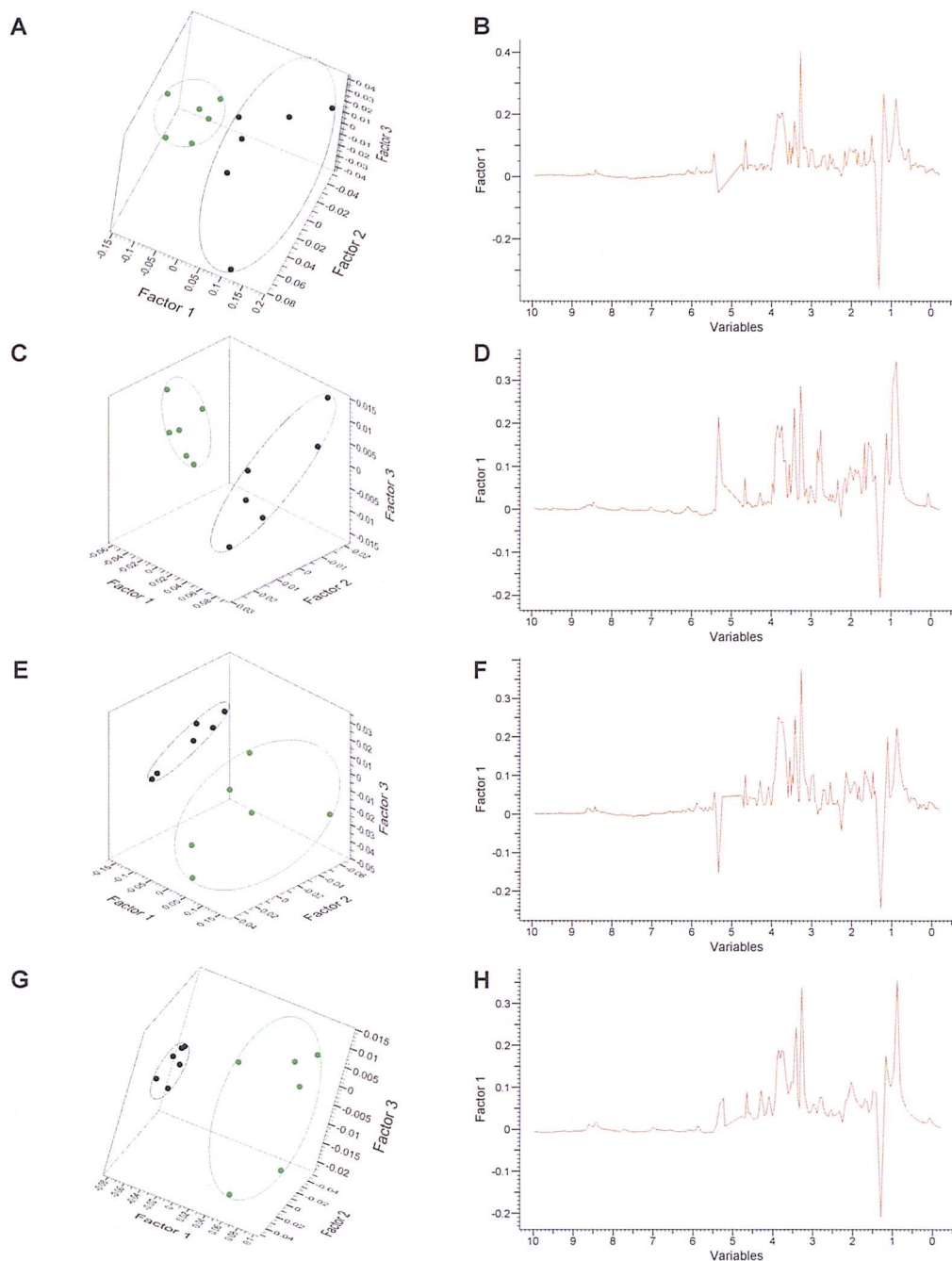


Figure 3-3. Principal components analysis of murine models and their controls.

Panels **A**, **C**, **E** and **G** denote scores plots, which place each sample in multidimensional space: axes labelled "factors 1-3" represent principal components 1-3. **B**, **D**, **F**, and **H** denote loadings plots, which show the spectral regions or "buckets", labelled "variables", corresponding to chemical shift of resonances from the spectra for each sample, and which contribute to the variation between datapoints on the scores plots. Loadings plots are shown from the first principal component only. Panels **A** and **B** compare BALB/c on HFD (green) to BALB/c on CD (black); **C** and **D** compare C3H on HFD (green) to C3H on CD (black); panels **E** and **F** compare C3H on CD (black) to BALB/c on CD (green); panels **G** and **H** compare C3H on HFD (black) to BALB/c on HFD (green).

Supervised PLS-DA models were then constructed. Correct class prediction was achieved with 100% accuracy for 6 of 7 comparisons of differing diet, strain and presence of the 263 mutation (Table 3-2).

Table 3-2. PLS-DA of each model compared to its control.

Model	Control	Components	Correct prediction of model	Correct prediction of control	Incorrect prediction of model (false positive)	No match	Q ²
BALB/c HFD	BALB/c CD	1	6/6	6/6	-	-	0.998
C3H HFD	C3H CD	1	6/6	6/6	-	-	0.898
Gena/263 HFD	Gena/263 CD	2	8/8	6/6	-	-	0.877
C3H CD	BALB/c CD	1	6/6	6/6	-	-	0.996
C3H HFD	BALB/c HFD	1	6/6	6/6	-	-	0.917
Gena/263 CD	Non-mut CD	1	6/6	6/6	-	-	0.982
Gena/263 HFD	Non-mut HFD	1	8/8	8/10	1	1	0.460

Metabolites contributing to the spectral regions correlating strongly (positively or negatively) with each model compared to its control are documented in **Table 3-3**. Lipid moieties, choline-related compounds, glucose, glycogen and a number of amino acid resonances (including glutamine, leucine, lysine and alanine) were found to contribute to class separation. In particular, PtC (with contributions from betaine and TMAO) was found to be a major contributor to the PLS-DA models. Accordingly, PtC was integrated using the CPMG spectra and divided by the tCho region for comparison with histological class. The use of tCho as a denominator allowed relative quantification of PtC without the potentially confounding dilutional effect of large changes in total lipid.

Table 3-3. Metabolites contributing to the discrimination of different murine models.

Model	Control	Primary discriminator	Major discriminators (order of decreasing influence on model)
BALB/c HFD	BALB/c CD	Lipid CH ₂	PtC, β-glucose, amino acids
C3H HFD	C3H CD	Lipid CH ₂	Lipid CH=CH, PtC, lipid CH ₂ CH ₂ CO
Gena/263 HFD	Gena/263 CD	Lipid CH=CH	Lipid CH ₃ , β-glucose, PtC
C3H CD	BALB/c CD	Lipid CH ₂	PtC, α and β glucose, amino acids
C3H HFD	BALB/c HFD	α and β glucose	Lipid CH ₂ , PtC, amino acids lipid CH ₃
Gena/263 CD	Non-mut CD	PtC	Lipid CH ₂ , α glucose, amino acids, glucose, PC, lipid CH=CH
Gena/263 HFD	Non-mut HFD	lipid CH=CH	lipid CH ₃ , α and β glucose, amino acids

Amino acid cluster includes resonances including leucine, alanine, lysine and glutamine.

In addition to the separation of each model from its control, PLS-DA models were constructed to enable the classification of a single sample from the total dataset. 54 samples were included in the total dataset and class membership established with 100% accuracy in all but two models (Table 3-4).

Table 3-4. Comparison of each group against all mice in the dataset.

Model	Diet	Components	Correct prediction of model	Correct prediction of non-fit	Incorrect prediction of model	No match	Q ²
BALB/c	CD	2	4/6	48/48	-	2	0.994
	HFD	5	6/6	48/48	-	-	0.996
C3H	CD	5	6/6	48/48	-	-	0.761
	HFD	3	6/6	48/48	-	-	0.998
Non-mut	CD	3	5/6	48/48	-	1	0.996
	HFD	3	10/10	44/44	-	-	0.996
Gena/263	CD	2	6/6	48/48	-	-	0.994
	HFD	2	8/8	46/46	-	-	0.977

Each PLS-DA model (from which 2 orthogonal factors had been removed, OSC) was constructed to separate the MR spectra obtained from a given mouse model from all other samples of all models in the total dataset.

3.5.3 Comparison between NMR spectroscopy and histological assessment

The distinction between simple steatosis and steatohepatitis is of importance for the development of models that reflect progressive disease in humans. Therefore PCA was performed on spectra from mice of all strains fed HFD and stratified by histological assessment of NAFLD. The samples were divided into histologically normal, steatotic and steatohepatitic groups on the basis of histological assessment of an adjacent slice of tissue. Steatohepatitis was defined in this study as the presence of hepatocyte ballooning +/- the presence of necroinflammatory infiltrate. The relationship between MR spectra and histological characteristics is shown in **Figure 3-4**. As disease severity increases, the lipid peaks increase in amplitude, compared to the water peak, while the glucose and glycogen resonances are reduced as steatosis worsens. All samples in the steatohepatitis group had evidence of hepatocyte ballooning. PCA demonstrated clustering of the groups, so PLS-DA was performed to assess the factors associated with category membership. Category membership (between histologically

normal, steatotic and steatohepatitic samples) using the leave-one-out algorithm was predicted with very high accuracy, as shown in **Table 3-5**.

Table 3-5. Comparison of histologically normal, steatotic and steatohepatitic samples.

Histological category	Components	Correct prediction of model	Correct prediction of non-fit	Incorrect prediction of model	No match	Q ²
Normal	3	5/6	24/24	-	1	0.997
Steatosis	3	6/6	24/24	-	-	0.996
Steatohepatitis	3	18/18	12/12	-	-	0.999

All samples from the HFD dataset. The PLS-DA model with OSC was constructed to separate the MR spectra obtained from a given category from all other samples of the other categories in the dataset.

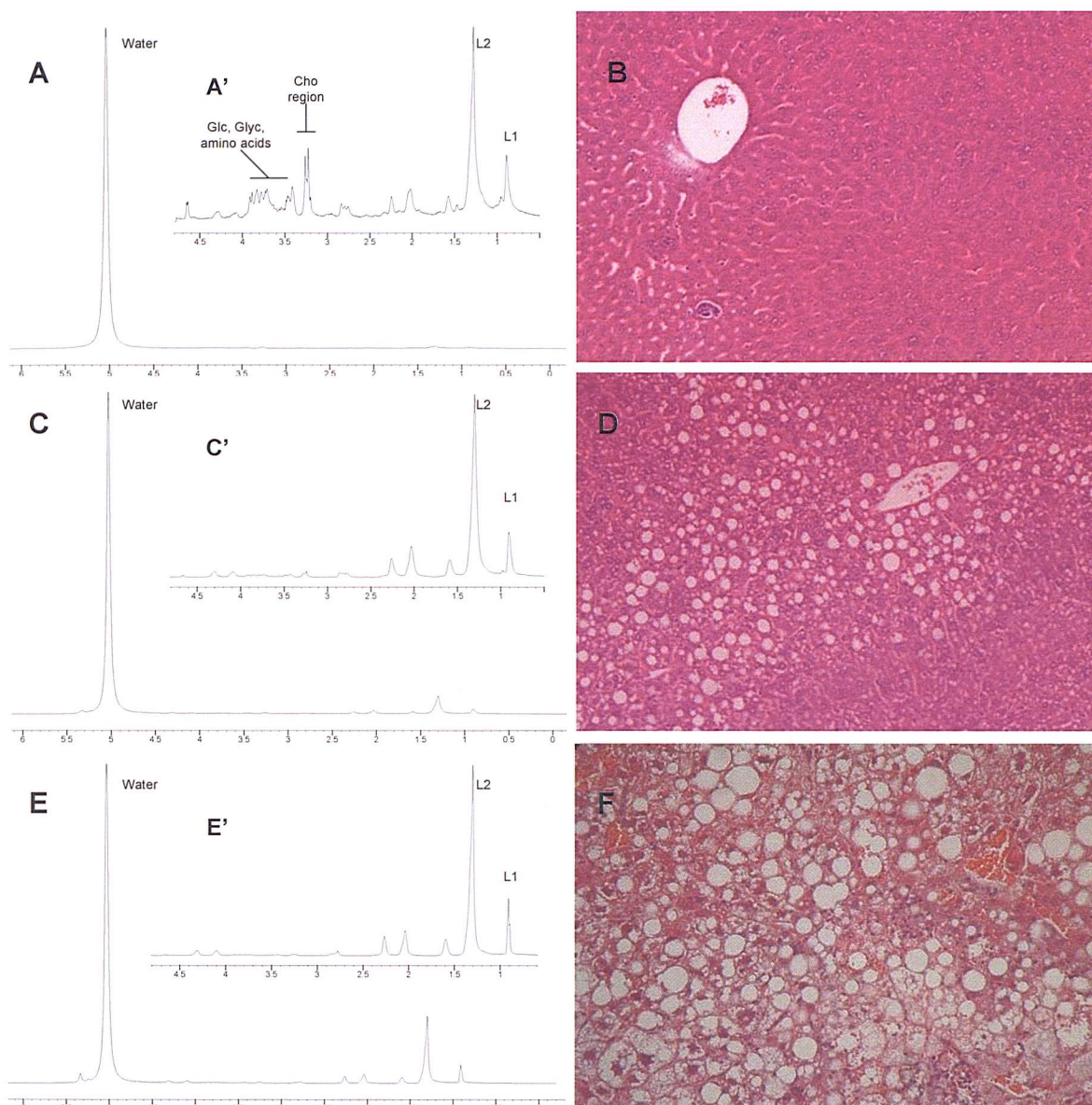


Figure 3-4. Representative ^1H MAS NMR spectra with paired histology.

NMR spectra are fully-relaxed pulse and collect sequences without and with water presaturation. Histology shows liver sections stained with haematoxylin and eosin at an original magnification of x200. Samples shown with normal architecture (A, A' and B), steatosis (C, C' and D) and steatohepatitis (E, E' and F). The “total signal” MR spectra (A, C, E) illustrate the ratio of water to other metabolites. The “lipid signal” MR spectra (A', C', E') amplify other major metabolite groups. The lipid constituents increase, while the tCho decreases with increasing disease severity. Characteristic histological features of steatosis (lipid droplets) and steatohepatitis (hepatocyte ballooning) from the corresponding tissue sample are shown in D and F.

The intrahepatic lipid level (median, interquartile range) differed significantly between samples histologically categorised as normal (1.96%, 1.02), steatosis (8.09%, 3.71) and steatohepatitis (10.56%, 6.08) (**Figure 3-5A**). There was a significant decrease in the PUFA:MUFA index between the normal (0.68, 0.09) and either steatotic (0.37, 0.12) or steatohepatitic (0.32, 0.09) samples. The PUFA:MUFA index did not differentiate between the steatotic and steatohepatitic groups (**Figure 3-5B**). The PUI was decreased in steatotic (3.40%, 0.95) and steatohepatitic mice (2.95%, 1.13) compared to normal mice (6.29%, 1.25) (Mann-Whitney U test, $p < 0.001$). Therefore, the increase in PUFA:MUFA index can be interpreted as consequent to a systematic decrease in PUFA with increasing disease severity. The resonances at $\delta 3.26$ ppm (PtC with TMAO and betaine) and $\delta 3.22$ - 3.23 ppm (PC and GPC) were found consistently to contribute to the difference between mouse classes on multivariate statistical modelling. tCho was significantly decreased in steatohepatitis (0.31%, 0.19) compared to steatosis (0.49%, 0.28) ($p = 0.01$) and in both compared to the histologically normal samples (1.61%, 0.69) ($p < 0.005$) (**Figure 3-5C**). Furthermore, PtC/tCho was significantly lower in the steatotic (33.7%, 16.8) and steatohepatitic animals (27.6%, 3.68) compared to normal animals (45.5%, 6.14) (**Figure 3-5D**). These findings are consistent with reports of low plasma PtC and high urinary choline oxidation products seen in 129S6 mice fed HFD (Dumas *et al.*, 2006).

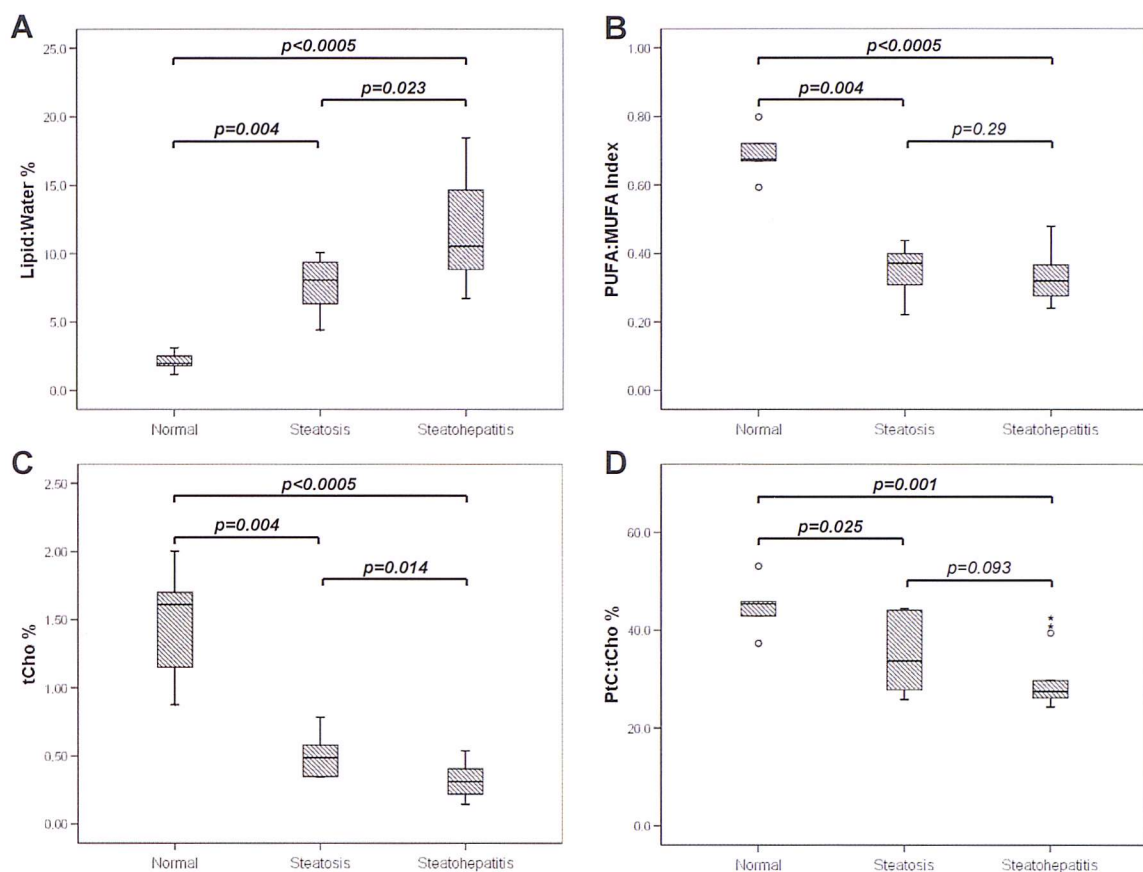


Figure 3-5. Differences in metabolite ratios (normal, steatotic and steatohepatitic liver).

Sample groups are histologically-defined. **A** shows the lipid:water ratio, consisting of the methylene functional group resonance expressed as a percentage of water. **B** shows the PUFA:MUFA ratio index. **C** shows the tCho level; **D** shows the PiC:tCho. *p*-values for the Mann-Whitney U test are stated (in bold for significance at the 95% level).

3.5.4 Relationship between intrahepatic lipid and obesity in murine models

Mouse mass plotted against the % lipid:water (**Figure 3-6A**) demonstrated that intrahepatic lipid increased exponentially with increasing mouse mass at time of culling ($R^2 = 0.80$, $p < 0.0005$). The % lipid:water was also compared to the histological assessment of steatosis and demonstrated a close relationship between steatosis score and % lipid:water (**Figure 3-6B**).

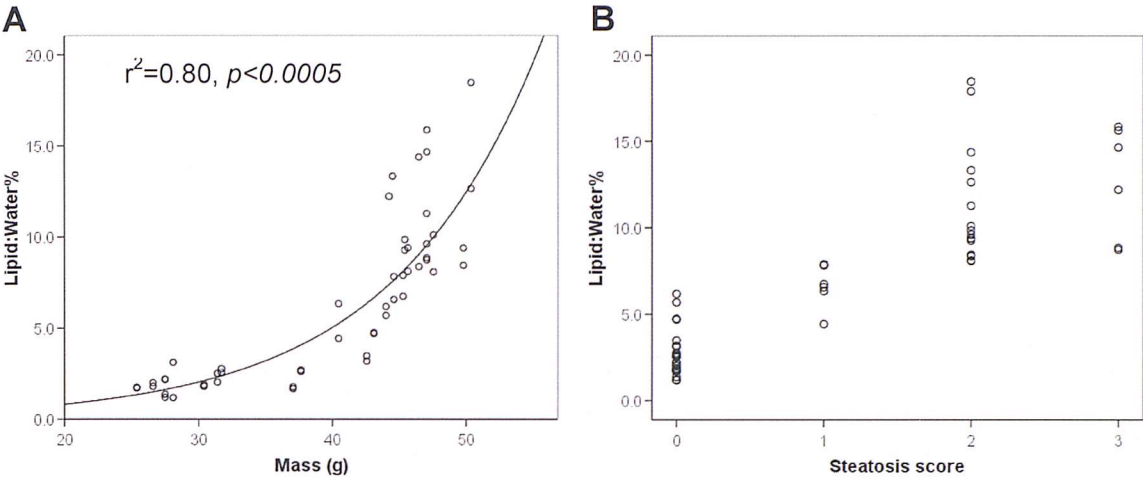


Figure 3-6. MR lipid content compared to mouse mass and histological steatosis score.

Scatter graphs showing the lipid:water ratio plotted against **A**, the mass of mouse in grams and **B**, the histological steatosis score.

3.6 Discussion

These data demonstrate that metabolic profiling of intact liver tissue using ^1H MAS NMR spectroscopy combined with PLS-DA is a powerful technique for the discrimination of NAFLD in animal models, differentiating by genetic (strain and the Gena/263 mutation), environmental (dietary) and histological factors. In particular, the first evidence is presented of accurate discrimination of histologically-defined steatosis and steatohepatitis using NMR of intact tissue. ^1H MAS NMR spectroscopy with PLS-DA also enables the separation and classification of different mouse strains on differing diets, despite normal histological appearances. Furthermore, it has been established that an exponential relationship exists between hepatic steatosis and obesity in these murine models.

In liver tissue, the “total signal” NMR spectra are dominated by water and lipid resonances. Such spectra allow the quantification of lipid, either in absolute terms, or as a proportion of the total signal in the spectrum. This marker of hepatic steatosis is a quantitative objective linear variable which compares favourably with the semi-quantitative subjective histological assessment of steatosis (**Figure 3-6**) (Szczepaniak *et al.*, 2005). This is an important observation as steatosis is prerequisite for the development of steatohepatitis and consequent fibrotic liver disease. In HFD-fed animals, there was a progressive significant increase in the % lipid:water from normal through steatotic to steatohepatic samples. This may represent increased uptake of fatty acids by the liver, decreased export or decreased catabolism (Anstee and Goldin, 2006). Additionally, defective peripheral fatty acid uptake shunts more fatty acids to the liver (Goldberg and Ginsberg, 2006). Intrahepatic lipid increased exponentially with animal mass, suggesting that fatty acid uptake peripherally was maximised in these mice at a weight approaching 40g. Thereafter, intrahepatic lipid accumulated in preference to peripheral uptake of fatty acids and deposition of triacylglycerol (TAG). Accumulated free fatty acids may be disposed of by esterification to triglycerides and by fatty acid oxidation producing oxidative stress and initiating inflammation (Yamaguchi *et al.*, 2007). While the traditional two-hit hypothesis proposed that the development of steatosis is followed by a second hit initiating

inflammation (Day and James, 1998), recent work by Yamaguchi and colleagues suggests that this model is now too simple, and that visible lipid deposition is less pathogenically relevant (Yamaguchi *et al.*, 2007). Differential fatty acid partitioning between esterification, leading to the accumulation of visible fat and oxidation of the invisible FFA, is more relevant to the pathogenesis. The use of NMR spectroscopy techniques in this study enables the determination of *total* lipid composition, which includes the assessment of the invisible hepatic FFA component.

The relative decrease in PUFA seen with increasing disease severity supports previous evidence that a decrease in PUFAs has been observed in the liver and blood of obese Zucker rats (Serkova *et al.*, 2006) and in the serum of human subjects with hepatic steatosis and steatohepatitis (Elizondo *et al.*, 2007; Puri *et al.*, 2007). A reduced PUFA:MUFA ratio has been considered to represent a surrogate marker of lipid peroxidation, associated with oxidative stress (Serkova *et al.*, 2006). Depletion of long chain PUFA (LC-PUFA) within the liver is thought to increase FA and TAG synthesis by upregulation of lipogenic genes via reduced downregulation of sterol regulatory binding protein (SREBP-1), and to decrease FA oxidation, including by a reduction of PPAR- α activation (Videla *et al.*, 2004).

PtC is an essential phospholipid (Li and Vance, 2008), existing as a membrane-bound macromolecule giving rise to a broad resonance (Millis *et al.*, 1999) within which the -N(CH₃)₃ functional group of trimethylamine-N-oxide (TMAO) and betaine (oxidation products of choline metabolism) are co-resonant (Rooney *et al.*, 2003). Measurement of choline levels demonstrates a decrease in choline-containing products in the obese models, and in those animals with steatosis and steatohepatitis, compared to histologically normal controls. Importantly, the tCho is significantly lower in steatohepatitis compared to simple steatosis. This is consistent with the observation that mice fed a choline-deficient diet develop hepatic steatosis and steatohepatitis (Walkey *et al.*, 1998). However, increasing lipid, which dominates the water-suppressed NMR spectrum, may also contribute to the reduction in tCho observed, by means of

a dilution effect. Therefore, the PtC resonance (also containing betaine and TMAO) was integrated in isolation and expressed as a ratio of the total choline region. PtC biosynthesis from choline and methionine is required for normal hepatic secretion of VLDL (Yao and Vance, 1988), and decreased PtC leads to accumulation of intracellular triglyceride (Noga *et al.*, 2002). Indeed, when comparing the PtC resonance to the total choline region, PtC is reduced compared to other choline-containing products in mice with hepatic steatosis and steatohepatitis compared to histologically normal livers. This is consistent with work on aqueous liver tissue extract in obese Zucker rats: a reduction in betaine at δ 3.26, compared to lean controls (Serkova *et al.*, 2006), has been shown to protect against alcoholic hepatic steatosis in experimental animals (Kharbanda *et al.*, 2007).

3.6.1 Use of multivariate pattern recognition statistics

^1H MAS NMR spectra contain a large number of resonances, both discrete and overlapping, the area of each of which is a continuous variable. One approach to data analysis is the integration of particular pre-defined resonances and comparison of such quantities between groups. This is an appropriate approach for deductive studies in which candidate markers of known processes have been identified. For inductive studies or top-down studies where at the outset differences are unknown, such pattern recognition statistical techniques include known variables (such as strain) and unknown variables (such as NMR spectral lineshape), which contribute to the model. In order to characterise samples of mouse liver in models of NAFLD rapidly, as in this study, such techniques powerfully discriminate between groups on the basis of NMR spectral changes correlating with each state.

In this study, PLS-DA has enabled the separation of different murine models of NAFLD from their control, and from each other. The discriminatory power of such classification has been assessed by cross-validation, by leaving out each sample in turn and constructing a model to determine the class membership of the excluded sample. This approach has enabled the correct prediction of the class membership of the spectrum from a given sample to be predicted both

from its control (100% accuracy in six of seven models) and from the whole dataset (100% accuracy in six of eight models). Accurate prediction of class membership suggests that this technique might be applied successfully to the screening of murine models to identify features of NAFLD.

3.6.2 Limitations and potential solutions

While ^1H MAS NMR spectroscopy allows the quantification of protons within functional groups of metabolites, it is not possible to quantify directly specific fatty acids, as this requires accurate measurement of fatty acid chain length and the position and number of functional groups within the chain. Nevertheless, the extent of saturation or (poly)unsaturation is of biological and clinical relevance.

The overlap of certain resonances (particularly small resonances near the larger lipid peaks) may reduce the accuracy of integration of those peaks. However, by dividing the spectra into small regions or “buckets”, quantification is objective and consistent. Curve-fitting software is used in the *in vivo* setting and enables quantification of small numbers of overlapping peaks. Newer approaches, such as “targeted profiling” may improve quantification of the multiple peaks found in complex tissue and biofluid samples *in vitro* (Weljie *et al.*, 2006).

The demonstration of accurate classification using PCA and PLS-DA, and the significant differences in metabolites concentrations as assessed by ^1H MAS NMR spectroscopy between histological groups demonstrate the power of the technique. However, validation in this study was by using a “leave-one-out” algorithm. If more samples had been available, the use of a separate validation set, not previously studied, would increase confidence in results further and would demonstrate the effectiveness of the technique for phenotyping models in practice. Moreover, the murine models used in this study covered the mild end of the spectrum of NAFLD severity: severe inflammation and fibrosis were not well represented. Murine models of more severe disease, such as a combined db/db (leptin resistant) methionine-choline deficient

diet model, would enable more effective stratification of histological disease severity over a wider range. One might expect even more florid NMR spectral differences to occur in such models, although the aetiological and pathological relevance of such extreme models to human disease is debatable (Anstee and Goldin, 2006).

3.7 Conclusions

In conclusion, this study has demonstrated the utility of ^1H MAS NMR spectroscopy of intact liver tissue in conjunction with multivariate modelling techniques for the investigation and classification of murine models of NAFLD on the basis of environmental, genetic and histopathological features. Class membership may be predicted with high accuracy and it is possible to distinguish reliably between samples, even where histological appearances are indistinguishable. PLS-DA enables the identification of biologically-relevant metabolites contributing to the differences between models. Future studies may employ these techniques to screen the output of programs generating murine models of NAFLD and other liver diseases. Greater subject numbers will facilitate the use of separate large training and test sets.

This study has demonstrated that a small number of resonances contribute reliably to the differentiation of the cohorts studied. Despite low field strength and the presence of motion artefact, tCho and a number of lipid resonances may be resolved in human liver *in vivo* (Fischbach and Bruhn, 2008), raising the possibility that ^1H MRS could provide discriminatory information in the context of NAFLD *in vivo*.

4 METABOLIC ANALYSIS OF HUMAN LIVER TISSUE IN HEPATITIS C *IN VITRO*

4.1 Abstract

Histological assessment of chronic hepatitis C-related liver disease provides a semi-quantitative morphological assessment of liver tissue. ^1H NMR spectroscopy enables metabolic profiling of liver tissue *in vitro*, which may provide insights into pathogenesis, with potential for translation into the *in vivo* setting.

The aim of this chapter was to investigate the metabolic profile of liver tissue in patients with chronic hepatitis C using ^1H NMR spectroscopy with multivariate factor analysis. Liver tissue from 47 patients with chronic hepatitis C was divided into two portions, one snap-frozen and analysed by ^1H MAS NMR spectroscopy at 11.7T, the other analysed using conventional histological techniques. Patients were stratified by fibrosis stage and HCV-genotype.

^1H MAS NMR spectroscopy was successfully performed using as little as 1.7mg of tissue. The resultant spectra were dominated by water and lipid peaks. Multivariate pattern-recognition analysis demonstrated characteristic NMR spectral differences by fibrosis stage and genotype, particularly from lipid and choline resonances. Total lipid was significantly increased in genotype 3 compared to genotype 1 and with increased fibrosis. Indices of lipid polyunsaturation, investigated previously in murine liver tissue, decreased with increasing fibrosis stage and, independently, with increasing steatosis.

Conclusion: characteristic differences in the metabolic profile of liver tissue in patients with chronic hepatitis C were discovered using high-resolution ^1H MAS NMR spectroscopy. Robust MR indices of lipid and choline composition were derived, which have the potential to be translated into the *in vivo* setting.

4.2 Introduction and aims

^1H NMR spectroscopy enables a snapshot metabolic profile to be obtained from a sample of interest and has been applied to intact tissue (**section 2.3**). Small-scale proof-of-principle studies have used this technique to assess metabolic perturbations in liver samples from patients with chronic liver disease (Duarte *et al.*, 2005; Martinez-Granados *et al.*, 2006). However, such studies were too small, or the patient groups too heterogenous to ascertain characteristic differences attributable to chronic liver disease.

Having optimised the parameters for ^1H MAS NMR spectroscopy of intact liver tissue in **Chapter 2** and applied the technique, in conjunction with multivariate pattern recognition analysis, to the characterisation of liver tissue in murine models of NAFLD in **Chapter 3**, the purpose of this study was to apply these techniques to tissue from liver biopsy from patients with chronic hepatitis C, in order to investigate characteristic metabolic differences that may complement histological assessment of liver biopsy or be translated into the *in vivo* setting.

Accordingly, the aims of this chapter were:

1. To apply high-resolution ^1H MAS NMR spectroscopy to investigate the metabolic profiles of liver tissue from patients with chronic hepatitis C.
2. To compare NMR spectral differences to histological assessment.
3. To derive simple and robust indices to stratify disease by fibrosis severity.

4.3 Materials and methods

4.3.1 Recruitment

47 patients with chronic hepatitis C were prospectively recruited from Imperial College Healthcare Trust from April 2006 to August 2007. Ethical approval for the study was obtained from the regional ethics committee in accordance with the 1975 Declaration of Helsinki. Patients were included if they were aged 18-65, had evidence of replicating hepatitis C-virus infection on HCV RNA testing, and had been referred for percutaneous liver biopsy for clinical indications.

Patients were excluded if they consumed >20g of alcohol per day, if they were obese (with a BMI >30kg/m²) or diabetic. Patients were also excluded if they were taking antiviral therapy; were co-infected with HIV or hepatitis B; were currently taking intravenous drugs, antihypertensive or lipid-lowering medications; had ongoing illness or had evidence of hepatic decompensation, defined by any of the following: jaundice, ascites, or hepatic encephalopathy.

4.3.2 Sample preparation

All patients underwent percutaneous liver biopsy by an experienced hepatologist using a 16-gauge Menghini needle. A portion (2-11 mm) was immediately snap-frozen in liquid nitrogen (within 3 minutes), then stored at -80°C and the remainder sent for routine clinical histological assessment. Biopsy samples for histology were fixed in formalin and embedded in paraffin wax, prior to staining with haematoxylin and eosin. All samples were scored according to the Ishak modified histological activity index (Ishak *et al.*, 1995) and by NASH Clinical Research Network semi-quantitative scoring system for cellular morphology, inflammation and fibrosis by an experienced hepatopathologist (Kleiner *et al.*, 2005). The patients were divided into three groups on the basis of the Ishak fibrosis stages as follows: group 1, mild fibrosis, stages 1-2; group 2, moderate fibrosis, stages 3-4; group 3, cirrhosis, stages 5-6. Freshly thawed liver tissue

was blotted to remove excess free fluid and placed into pre-cooled XC4 Kel-F sealing cells (Doty Scientific Inc., Columbia, South Carolina, USA) prior to examination.

4.3.3 ^1H MAS NMR

^1H MAS NMR spectroscopy experiments were performed as described in **Chapters 2 and 3** (Cobbold *et al.*, 2008a). Briefly, an MAS probe was interfaced with a JEOL Eclipse 500+ spectrometer and an 11.7T superconducting magnet (**section 2.6.1**). Samples were spun at 4000Hz and kept at 4°C to minimise sample degradation during data acquisition (**section 2.7**). Sequences specific to this study included a pulse and collect sequence (pulse angle 90°, receiver gain 5, repetition time (TR) 10s, 32 data collects), to derive a fully-relaxed spectrum without suppression of the water resonance and a pulse and collect with water presaturation sequence (pulse angle 90°, receiver gain 10, TR 5s, 128 data collects) produced a spectrum dominated by lipid. 32K data points were acquired for each spectrum with a spectral width of 30ppm (Mahon *et al.*, 2004a). The overall time for data acquisition was 28 minutes.

4.3.4 Data analysis and interpretation

Spectra were processed using KnowItAll® Informatics System v7.8 (Bio-Rad, Philadelphia, USA) as previously described in **section 3.4.3** (Cobbold *et al.*, 2008a). Specifically, MR spectra were referenced to the methylene lipid ($-\text{CH}_2$) peak at $\delta 1.30\text{ppm}$ and resonances were assigned according to the published literature (Bollard *et al.*, 2000; Duarte *et al.*, 2005; Martinez-Granados *et al.*, 2006; Rooney *et al.*, 2003). Analysis was divided into hypothesis-generating multivariate analysis and biologically-relevant conventional analysis of specific metabolites and regions.

4.3.4.1 *Multivariate data analysis*

Multivariate data-reduction techniques were applied in order to use information from the whole spectrum, and to determine which peaks contributed to the metabolic variation between individual samples and between groups of samples.

Unsupervised multivariate factor analysis was performed using principal components analysis (PCA) to assess clustering and the presence of outliers. Supervised analysis was then performed using partial least squares discriminant analysis (PLS-DA) to investigate factors associated with disease severity and genotype, with the Pirouette v4.0 software package (Infometrics, Washington, USA) as described in **section 3.4.4** (Cobbold *et al.*, 2008a).

4.3.4.2 *Conventional statistical methods*

SPSS v16 (SPSS Inc. Chicago, USA) was used for analysis. Clinical and biological indices were expressed as median values with interquartile range (or range). MRS-derived indices were compared using the Mann-Whitney U test for pair-wise comparisons. Correlation was performed using the Spearman Rho test, with fibrosis stage (0-6) approximating a linear variable. For the regression of dependent variables, residual plots were inspected for evidence of non-normality or non-linearity of effect and the dependent and independent variables were transformed appropriately.

4.4 Results

4.4.1 Patient Characteristics

The demographic and clinical characteristics of the 47 patients are displayed in **Table 4-1**. 35 of 47 patients (75%) were Europoid, 5 were South Asian (11%), 3 were of Middle-Eastern or of Arab East African origin (6%), 2 were black African (4%) and 2 were East Asian (4%). 51% of patients were infected with genotype 1, 6% with genotype 2, 32% with genotype 3 and 11% with genotype 4. The median length of biopsy samples analysed for histology was 30mm (IQR 14mm), while an average of 6mg (3.6mg) of tissue per patient was analysed using ^1H MAS NMR spectroscopy.

Table 4-1. Patient characteristics

	Total	Fibrosis group			Genotype		
		1	2	3	1	3	Other (2,4)
Number	47	19	19	9	24	15	8
Sex M/F	29/18	8/11	13/6	8/1	14/10	11/4	4/4
Age (years)	46 (13)	41.5 (19.5)	47 (10)	47 (11)	45 (14)	46 (10)	48.5 (24)
Genotype (no. 1/3)	24/9	12/4	7/8	5/3	N/A	N/A	N/A
Bx length, mm (range)	30 (10-49)	32 (11-43)	27.5 (10-49)	24 (10-48)	30 (10-48)	21 (10-49)	33 (25-45)
Mass sample for NMR, mg (range)	5.8 (1.7-11.4)	5.5 (2.0-10.8)	5.9 (1.7-11.4)	5.8 (2.3-7.7)	5.95 (1.7-8.6)	6.0 (2.3-11.4)	4.8 (2.0-7.3)
BMI	24.0 (4.1)	24.2 (5.3)	23.5 (3.2)	25.8 (2.7)	24.0 (4.4)	24.2 (3.1)	23.8 (3.7)
ALT, iu/L [0-40]	61.5 (66)	45.0 (32.0)*	75.5 (46.5)*	160.0 (133.0)*	59.0 (42.0)†	86.5 (131.0)†	60 (78.3)
AST, iu/L [0-40]	44.5 (61.3)	32.0 (17.0)*	46.5 (45.0)*	118.0 (105.0)*	43.5 (23.8)†	79.5 (98.8)†	46.0 (104.5)
Platelets, x10⁹/L [130-450]	253.0 (73.5)	291.5 (132.8)*	252.5 (37.5)*	185.0 (81.5)*	253.0 (101.0)	253.0 (97.2)	253.0 (90.0)
Total Cholesterol, mmol/L [<6.5]	4.3 (1.19)	4.6 (1.1)	4.1 (0.7)	4.8 (1.5)	4.6 (1.3)	4.2 (0.3)	
Triglycerides, mmol/L [<2.5]	1.0 (0.40)	1.0 (0.2)	1.1 (0.6)	0.9 (0.4)	1.0 (0.40)	0.95 (0.73)	

Figures are median (interquartile range) unless otherwise specified. M, male; F, female; Bx, biopsy; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase. * significant at the p=0.05 level, using the Kruskal- Wallis Test. †, significant at the p=0.05 level using the Mann- Witney U test for pairwise comparisons.

4.4.2 MR Characterisation

A representative water-suppressed ^1H MAS NMR spectrum from intact human liver tissue is shown in **Figure 4-1**, illustrating a number of specific lipid resonances including fatty acid functional groups. The total choline region ($\delta 3.18$ - $\delta 3.28$ ppm) (tCho) contains peaks representing choline, phosphocholine, glycerophosphocholine and phosphatidylcholine, with contributions from trimethylamine-N-oxide (TMAO) and betaine.

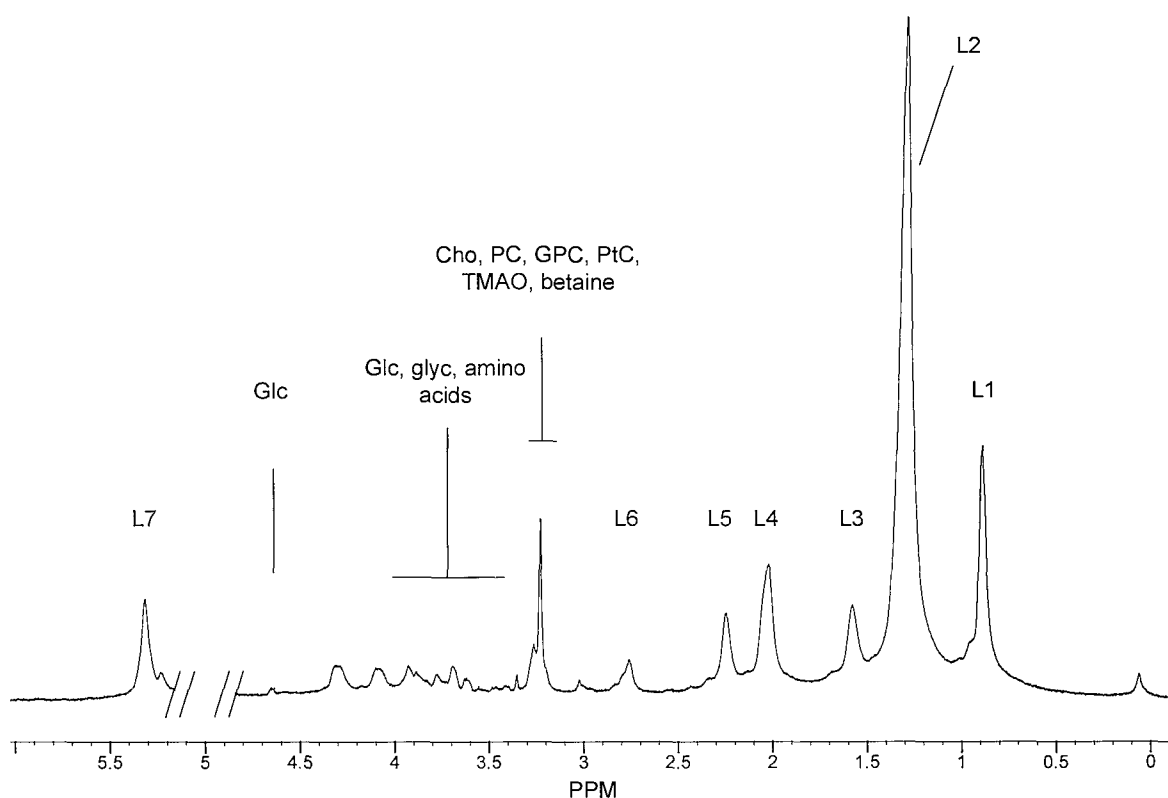


Figure 4-1. ^1H MAS NMR spectrum from human liver biopsy tissue.

Fully-relaxed pulse and collect ^1H MAS NMR spectrum with water suppression, of intact liver from a liver biopsy specimen from a patient with hepatitis C, genotype 3, moderate fibrosis. The residual water resonance at $\delta 4.8$ - 5.2 was removed in the region indicated. Key: L1, lipid CH_3CH_2 (methyl); L2 lipid $(\text{CH}_2)_n$ (methylene); L3 lipid $\text{CH}_2\text{CH}_2\text{CO}$ (methylene to L5); L4 lipid $\text{CH}=\text{CHCH}_2\text{CH}_2$ (allylic); L5 lipid $\text{CH}_2\text{CH}_2\text{CO}$ (α -methylene to carboxyl); L6 lipid $\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}$ (diallylic- "PUFA"); L7 lipid $\text{CH}=\text{CH}$ (n -ene group); Cho, choline; PC, phosphocholine; GPC, glycerophosphocholine; PtC, phosphatidylcholine; TMAO, trimethylamine-N-oxide; Glc, glucose; Glyc, glycogen..

4.4.2.1 *Multivariate pattern-recognition analysis*

There was clustering of data according to fibrosis stage and genotype on PCA following integration of “bins”. The major metabolites contributing to the differences between mild and moderate to severe fibrosis and between genotypes 1 and 3 are shown in **Table 4-2**. Fatty acyl functional groups and choline-containing compounds were seen to contribute substantially to the PLS-DA models separating samples and formed the basis of indices derived for subsequent analysis. Applying the leave-one-out cross-validation algorithm to the *in vitro* cohort, fibrosis group and genotype were correctly predicted in 46/47 cases (**Table 4-3**).

Table 4-2. Metabolites contributing to differences between groups

Metabolite	Chemical shift	Rank/total bins	Coefficient
Fibrosis: Mild from moderate and cirrhosis			
GPC, PC	3.21-3.25	1	-6.74
Lipid $-\text{CH}_3$	0.81-0.93	2	-6.55
Lipid $-\text{CH}_2$	1.19-1.39	3	5.48
Glycogen	3.67-3.73	4	-3.43
PtC, TMAO, Betaine	3.25-3.28	5	-3.25
Lipid $-\text{CH}_2\text{CH}_2\text{CH}=\text{CH}$	1.95-2.10	6	-3.13
Ala, Lys, Gln, GPC	3.75-3.81	7	-2.92
Lipid $-\text{CH}_3$	0.75-0.81	8	-2.86
Glycogen, PC, Gly, Thr	3.59-3.65	9	-2.41
Lipid $-\text{CH}_2\text{CH}_2\text{CO}$	1.65-1.70	10	-2.09
Diallylic lipid	2.74-2.79	50	-0.82
Genotype 1 from 3			
GPC/PC	3.21-3.25	1	-4.01
Lipid CH_3	0.81-0.93	2	-3.99
Lipid CH_2	1.22-1.40	3	3.89
Lipid CH_2	1.16-1.21	4	-2.42
Glycogen	3.67-3.73	5	-2.08
Lipid $-\text{CH}_2\text{CH}_2\text{CH}=\text{CH}$	1.95-2.10	6	-1.78
Lipid CH_3	0.76-0.81	7	-1.65
Ala, Lys, Gln, GPC	3.75-3.81	8	-1.61
PtC, TMAO, Betaine	3.25-3.28	9	-1.57
Glycogen, PC, Gly, Thr	3.59-3.65	10	-1.45
Diallylic lipid	2.74-2.79	83	-0.65

Abbreviations: GPC, glycerophosphocholine; PC, phosphocholine; PtC, phosphatidylcholine; TMAO, trimethylamine-N-oxide, Ala, alanine; Lys, lysine; Gln, glutamine; Gly, glycine; Thr, threonine. Diallylic lipid is $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$ functional group, PUFA.

Table 4-3. Cross-validation of PLS-DA models for prediction of fibrosis and genotype

Category	Correct prediction of category	Correct prediction of non-fit	Incorrect prediction of category	No match	Q ²
<i>Fibrosis</i>					
Mild (stage 0-2)	19/19	27/28	0	1	0.994
Moderate (stage 3-4)	19/19	27/28	0	1	0.996
Cirrhosis (stage 5-6)	8/9	38/38	0	1	0.994
<i>Genotype</i>					
1	24/24	14/15	0	1	0.998
3	14/15	24/24	0	1	0.998

Leave-one-out cross-validation of PLS-DA three-component models for the prediction of fibrosis category and genotype.

4.4.2.2 *Analysis of lipid composition*

On the basis of multivariate analysis and from previous studies, indices were derived to assess specifically the percentage total lipid, the total choline to lipid ratio, the degree of fatty acid saturation (saturation index, SI) and the degree of polyunsaturation (polyunsaturation index, PUI) (Cobbold *et al.*, 2008a; Johnson *et al.*, 2008). The indices are defined in **Table 4-4**.

Table 4-4. Derivation of indices

Index	Water suppression	Definition
Total lipid (%)	No	$100 * (\text{methyl} + \text{methylene} / \text{water} + \text{methyl} + \text{methylene})$
tCho:Lipid (%)	Yes	$100 * (\text{tCho} / \text{methyl} + \text{methylene} + \text{allylic} + \text{diallylic})$
SI (%)	Yes	$100 * 1 - (\text{allylic} + \text{diallylic} / \text{allylic} + \text{diallylic} + \text{methyl} + \text{methylene})$
PUI (%)	Yes	$100 * \text{diallylic} / \text{diallylic} + \text{allylic} + \text{methyl} + \text{methylene}$

Abbreviations: tCho, total choline; SI, saturation index; PUI, polyunsaturation index.

Indices of lipid composition were stratified by severity of fibrosis and displayed in **Figure 4-2**. There was a positive correlation between total lipid and fibrosis stage, $r=0.43$, $p=0.003$. SI increased with fibrosis, $r=0.58$, $p<0.0005$, while the choline to lipid ratio decreased with increasing fibrosis, $r=-0.51$, $p<0.0005$, and the PUI was also negatively correlated, $r=-0.55$, $p<0.0005$.

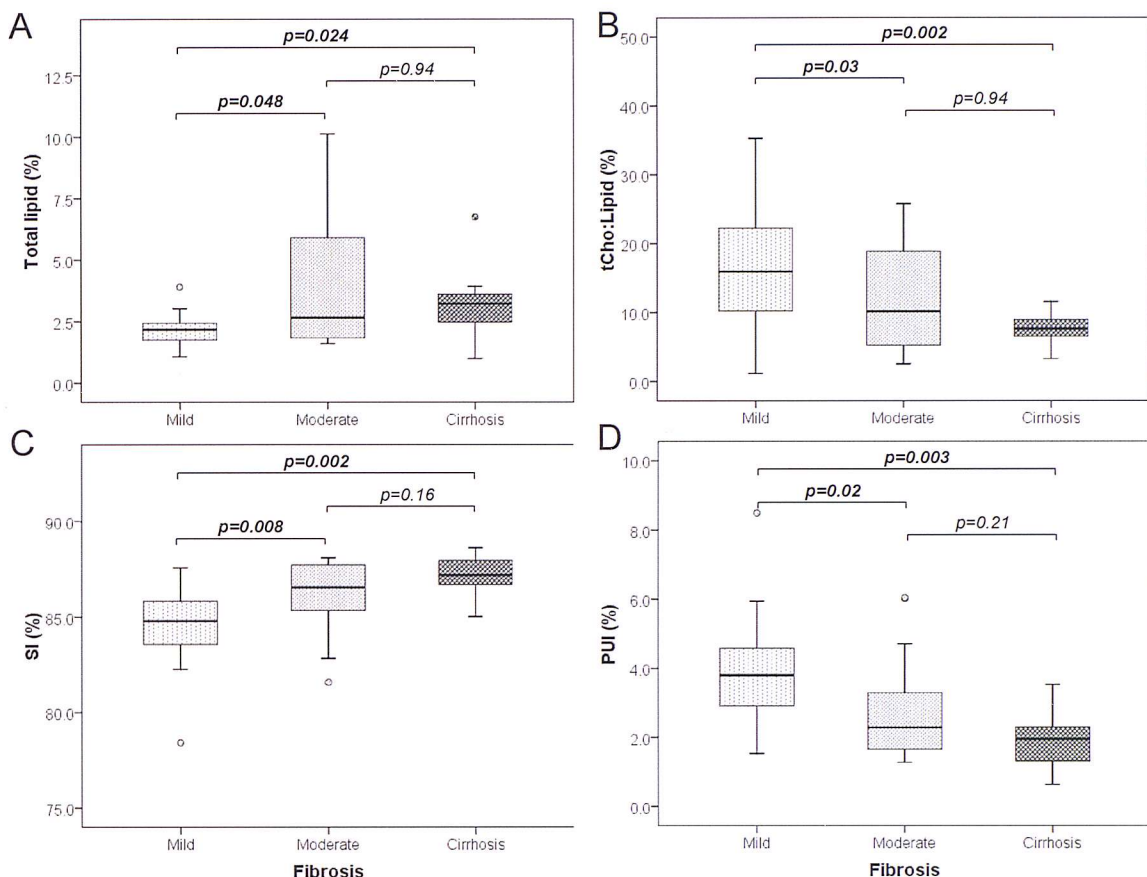


Figure 4-2. *In vitro* ^1H MAS NMR spectral indices plotted against fibrosis stage.

Categories are mild fibrosis (F1-2), moderate fibrosis (F3-4) and cirrhosis (F5-6). “Box and whisker” plots represent the median (horizontal line) and interquartile range (box) with the range of values up to 1.5 times the IQR within the range of each of the bars. Outliers are plotted individually beyond the bars. Significant *p*-values from the Mann-Whitney U test are displayed in bold, non-significant differences in normal weighted text. **A**, percentage total lipid: median (IQR) increased from 2.18% (0.74) through 2.85% (0.76) to 3.22% (1.54) with increasing severity of fibrosis group. **B**, the total choline to lipid ratio: decreased from 15.97% (13.54) through 10.19% (14.01) to 7.66% (3.28); **C**, the saturation index increased from 84.98% (2.15) through 86.55% (2.79) to 87.19% (2.09). **D**, the polyunsaturation index decreased from 3.54% (1.71), through 2.29% (1.78) to 1.95% (1.72).

Clear differences in lipid load and composition were also notable between patients with hepatitis C genotype 1 and genotype 3 (**Figure 4-3**). The total lipid content and SI were higher in those with genotype 3, compared with genotype 1. The tCho did not differ significantly between groups, but the PUI was lower in genotype 3 than genotype 1. From **Table 4-1**, it can be seen that patients with genotype 3 disease had more severe disease than those with genotype

1 (as assessed by histological fibrosis stage and aminotransferase levels). In order to determine whether the differences in total lipid were dependent on genotype or severity, the total lipid and PUI were plotted by severity and stratified by genotypes 1 and 3 (**Figure 4-4**). This showed that, in genotype 1, total lipid increased with histological fibrosis stage (Spearman rho $r=0.53$, $p=0.01$), while in genotype 3, there was no association with fibrosis stage ($r=-0.01$, $p=0.74$). However, PUI correlated with severity for both genotype 1 ($r=-0.42$, $p=0.04$) and genotype 3 ($r=-0.54$, $p=0.04$).

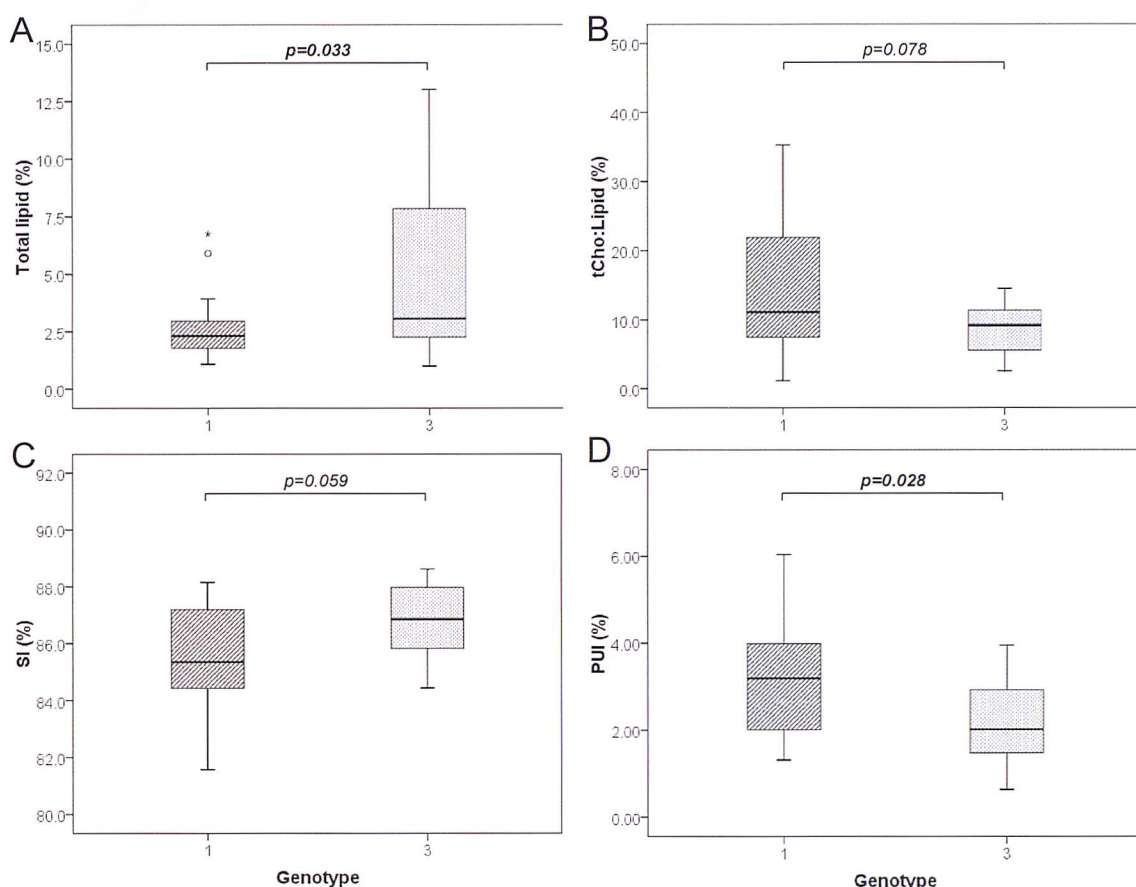


Figure 4-3. ^1H MAS NMR spectroscopy-derived indices, plotted by genotype.

“Box and whisker” plots represent the median (horizontal line) and interquartile range (box) with the range of values up to 1.5 times the IQR within the range of each of the bars. Outliers are plotted individually beyond the bars. Significant p -values from the Mann-Whitney U test are displayed in bold, non-significant differences in normal weighted text. A, median total lipid was significantly lower in genotype 1, 2.17% (1.36) compared to genotype 3, 3.07 (5.87); B, the median total choline to lipid ratio higher in genotype 1, 11.60 (15.40) versus 3, 9.18 (6.31); C, the median saturation index was lower in genotype 1, 85.7 (3.04) than 3, 86.9 (2.31); D, the polyunsaturation index was significantly higher in genotype 1, 3.18% (2.13) than 3, 2.01% (1.66).

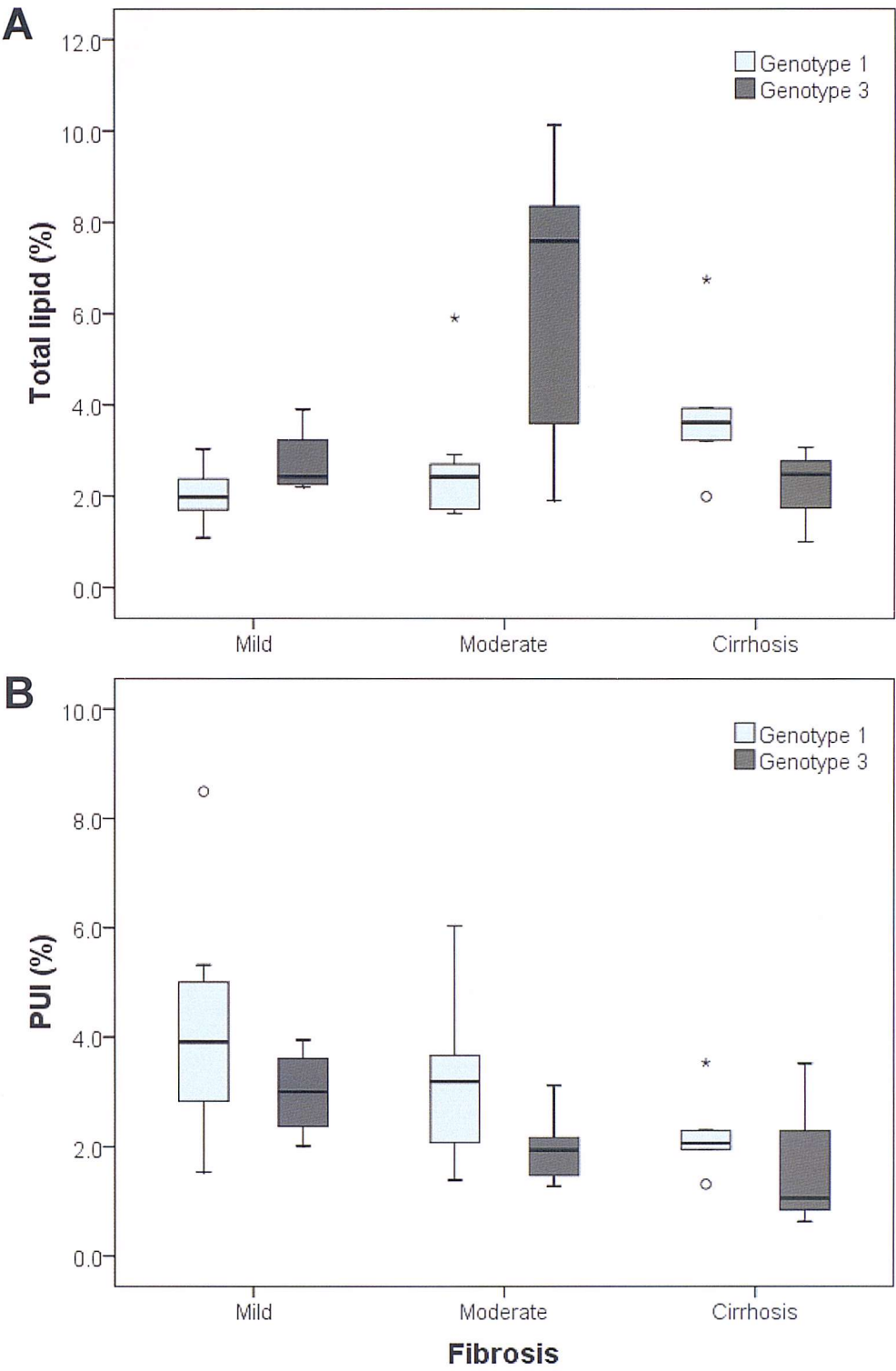


Figure 4-4. Total lipid and PUI stratified by severity of fibrosis and genotype

“Box and whisker” plots represent the median (horizontal line) and interquartile range (box) with the range of values up to 1.5 times the IQR within the range of each of the bars. Outliers are plotted individually beyond the bars. A, total lipid; B, polyunsaturation index.

Multiple linear regression was performed to assess the relative contribution of fibrosis, necro-inflammation and lipid load (lipid droplets as a percentage of hepatocytes) as assessed histologically on the NMR-derived indices (**Table 4-5**). The ordinal histological scores approximated a linear variable. On univariate and multivariate analysis, both fibrosis score and steatosis score significantly influenced PUI, but necro-inflammatory score had no effect. The magnitude of the effect of fibrosis on PUI was greater than that of steatosis in this cohort of patients, although this cohort was not recruited or stratified primarily by steatosis.

Table 4-5. Linear regression of histological features on the MRS-derived indices.

Model	Variable	Coefficient	R ²	Significance
PUI			0.413	
	Fibrosis stage (0-6)	-0.472		<0.0005
	Steatosis (0-3)	-0.285		0.028
SI			0.428	
	Fibrosis stage (0-6)	-0.503		<0.0005
	Steatosis (0-3)	-0.315		0.011
CLR			0.420	
	Fibrosis stage (0-6)	-0.449		0.001
	Steatosis (0-3)	-0.399		<0.0005

Significant values are in bold type. Candidate variables were fibrosis stage, steatosis grade and necro-inflammation. Necro-inflammatory score was not a significant contributor to any of the models.

4.5 Discussion

High resolution ^1H MAS NMR spectroscopy of liver biopsy samples from patients with chronic hepatitis C provided metabolic profiles showing clear differences according to disease severity and genotype. Cross-validation of the PLS-DA model showed that the fibrosis category or genotype could be predicted correctly in 46 out of 47 cases and that these differences were predominantly due to changes in lipid and choline resonances. Total lipid and the degree of fatty acid saturation increased with increasing disease severity, and in genotype 3 compared with genotype 1. The extent of fatty acid polyunsaturation decreased independently with increased fibrosis and with increasing steatosis. The total choline to lipid ratio also decreased with increasing disease severity. Differences in indices according to genotype (1 or 3) should be interpreted in the context of the distribution of genotypes according to histological severity (**Table 4-1**). In this cohort, patients with genotype 3 had more severe disease than those with genotype 1: patients with genotype 3 were more likely to have moderate or severe disease (assessed histologically) than those with genotype 1 and transaminases were also higher. This may partly explain why the table of metabolites contributing to differences in fibrosis severity is similar to that differentiating genotypes 1 and 3 (**Table 4-2**). There is no clear evidence that the genotype has a substantial effect on the rate of progression of disease, although some studies have suggested that genotype 1b is associated with more aggressive, rapidly progressive disease (Zein, 2000). The explanation for increased disease severity in those with genotype 3 may lie in the duration of infection, although this information was unavailable for the current study. Certainly, (south) Asians with genotype 3 disease have been found to have a higher fibrosis stage at presentation than non-Asians and this has been linked to infection earlier in life among this group (Freshwater *et al.*, 2008).

The cohort of 47 biopsies from well-characterised patients with chronic hepatitis C, analysed using ^1H MAS MRS with multivariate pattern recognition statistics has enabled the observation of characteristic metabolic differences with fibrosis stage and genotype. This represents the largest number of patients with chronic liver disease studied using this approach. Proof-of-

principle *in vitro* studies have applied high resolution MRS to human liver tissue from six organs for transplantation pre-and post transplant (Duarte *et al.*, 2005) and to 17 patients with chronic liver disease of differing aetiologies (Martinez-Granados *et al.*, 2006). These initial studies demonstrated the range of metabolites observable in human liver tissue, but owing to their small size and the heterogenous patient groups, robust characteristic changes with disease state were not seen.

4.5.1 Biological relevance of indices

Total intrahepatic lipid was found to increase with increasing fibrosis stage. The presence of steatosis is associated with an increased rate of progression of fibrosis (Adinolfi *et al.*, 2001), which may leading to a higher prevalence of steatosis with increasing fibrosis stage. Total lipid is also increased in those patients with genotype 3 compared with those patients with genotype 1. Stratification of data by both histological fibrosis severity and genotype categories (**Figure 4-4**) showed that, although total lipid was higher in genotype 3 than in genotype 1 overall, in genotype 1 there was a good correlation with fibrosis stage, while in genotype 3, there was no such correlation. This suggests that in patients with genotype 3, HCV-related “viral” steatosis is the dominant effect, rather than a constitutive, host-related process. Moreover, hepatic steatosis is strongly influenced by genotype, rather than simply being a correlate of disease severity. To substantiate these relationships, larger prospective longitudinal studies would need to be performed with acquisition of data including the duration of infection and consideration of race.

Virological mechanisms for steatosis have been proposed, including inhibition of the microsomal triglyceride transfer protein by HCV core protein (Perlemuter *et al.*, 2002), the activation by cleavage of sterol regulatory binding proteins (Waris *et al.*, 2007), and the presence of genotype 3-specific core protein residues (Hourieux *et al.*, 2007), as discussed in detail in **section 1.5**. Mechanisms of metabolically mediated steatosis in HCV include increased insulin resistance (Hui *et al.*, 2003), which is associated with increased levels of TNF α in animal studies (Shintani *et al.*, 2004). Fasting insulin assays were unavailable in this study, but

would have enabled more detailed analysis of the contributions of “viral” and “metabolic” aspects of HCV-associated hepatic steatosis.

The hepatic total choline concentration decreased with increasing fibrosis. The dominant components of the choline region, GPC, PC and PtC, are prominent contributors to the PLS-DA models separating NMR spectra by fibrosis severity and by genotype. Total choline also decreases with increasing hepatic lipid, in keeping with decreased phosphocholine in NAFLD observed using chromatography-based lipidomic analysis (Puri *et al.*, 2007). Studies have shown PtC to be required for normal hepatic secretion of VLDL (Yao and Vance, 1988) and decreased PtC leads to accumulation of intracellular triglycerides (Noga *et al.*, 2002). In addition, mice fed a choline/methionine deficient diet are known to develop hepatic steatosis and steatohepatitis (Walkey *et al.*, 1998). However, as studies are carried out on a fixed volume of tissue, increasing steatosis may contribute to decreased total choline measurement, owing to a dilutional effect of higher lipid levels.

There was a decrease in PUFA indices and, conversely, an increase in saturation with increasing fibrosis stage (**Figure 4-2**). PUI correlated with fibrosis severity, both in those with genotype 1 and in genotype 3, in contrast to total lipid measurement (**Figure 4-4**). This supports PUI being predominantly a marker of disease severity, rather than a marker of the steatotic effects of genotype. Regression of histological features demonstrated that reduced PUI was independently associated with increased fibrosis and steatosis, but not with necroinflammation (**Table 4-5**). PUFAs are important cell membrane constituents, incorporated into phospholipids and contributing to fluidity and cell membrane function (Cabre and Gassull, 1996). The degree of lipid polyunsaturation is of particular interest in chronic liver disease and has been seen to be reduced in animal studies using MRS *in vitro* (Cobbold *et al.*, 2008a; Serkova *et al.*, 2006), and in human studies of NAFLD using chromatography techniques (Puri *et al.*, 2007). Moreover, studies of hepatic cell lines have shown that addition of PUFA inhibit HCV RNA replication (Kapadia and Chisari, 2005). The decrease in PUFA seen in our study may be a result of reduced dietary essential fatty acids and/or a reduction in desaturase activity consequent to liver

insufficiency *per se* (Cabre and Gassull, 1996; Puri *et al.*, 2007). However, as discussed in **section 1.5.3**, diseased liver is subject to oxidative stress, which in the case of CHC, is associated with the HCV viral load and inflammation (Okuda *et al.*, 2002). PUFAs are consumed by oxidative and peroxidative processes in conditions of oxidative stress and consequent products, such as malondialdehyde (MDA) and 4-hydroxyl-2,3-nonenal (HNE) adducts, have been associated histologically with viral chronic liver disease, correlating with fibrosis score and disease activity (Paradis *et al.*, 1997b). Proteomic profiling of liver biopsy samples suggested impairment of fatty acid oxidation and response to oxidative stress with severe fibrosis compared with mild fibrosis (Diamond *et al.*, 2007). These studies suggest that the ability to monitor hepatic PUFA non-invasively may be of considerable biological importance for subsequent studies of disease progression and intervention.

4.5.2 Limitations

^1H MAS NMR spectroscopy enables the quantification of total lipid, but also conveys information on lipid composition in addition to other metabolites. Indices of fatty acid saturation and polyunsaturation are robust and of clinical and biological importance, but the information is limited, as individual fatty acid species are not directly quantifiable.

These experiments were performed on a small portion of clinical liver biopsy specimens (1.7-11.4mg) likely resulting in sampling variability. However, the portion used for ^1H MAS NMR spectroscopy was from the same core as that sent for histology, so was spatially related to the reference standard.

As the water-suppressed NMR spectra are dominated by lipid resonances, quantification of any resonance as a proportion of the total spectra integral will be influenced by the lipid load, leading to a dilutional or “bulk” effect. Correction for sample mass may allow the derivation of molar quantities of metabolites, but does not correct for increased steatosis. Water unsuppressed MR spectra allow absolute quantitation, although the presence of a large water peak interferes

with neighbouring resonances. While CPMG sequences preferentially suppress signal from macromolecules including lipids, the effects on other metabolites are variable, so absolute quantification requires knowledge of T_2 values. Novel approaches to spectral processing may allow the editing out of lipid resonances, so a proportion of the tissue excluding lipid might be assessed (Weljie *et al.*, 2006). Pragmatically, however, where the mass or volume of tissue is constrained, MRS data is easily expressed as ratios of the metabolites of interest. However, interpretation of these ratios should include consideration of bulk effect.

For the purposes of establishing the pathological significance of lipid composition indices, subgroup analysis by race and genotype was underpowered to make firm conclusions. In addition, fasting insulin measurements were not obtained prospectively, so the relationship between insulin resistance, genotype and hepatic steatosis could not be explored in this cohort. The effects of insulin resistance and race will, however, be investigated further in **Chapter 5**.

4.6 Conclusion

This study has used high resolution ^1H MAS NMR spectroscopy of liver biopsy samples to examine the metabolic profile in patients with chronic hepatitis C. Lipid quantity and composition accounted for much of the NMR spectral differences between groups: an increase in total lipid and a decrease in lipid polyunsaturation was observed with increasing hepatic fibrosis, while increased intrahepatic lipid was also observed in patients with genotype 3. These robust NMR indices demonstrate potential to be translated to the *in vivo* setting.

5 NON-INVASIVE ASSESSMENT OF LIPID COMPOSITION IN PATIENTS WITH HEPATITIS C

5.1 Abstract

Hepatic steatosis is an important factor in the pathogenesis, progression and response to treatment in hepatitis C. Histological assessment of hepatic lipid is limited by sampling variability and the semi-quantitative nature of scoring systems. ^1H MRS enables metabolic profiling of liver *in vivo*, providing a potentially non-invasive approach.

The aim of this study was to investigate lipid composition in patients with chronic hepatitis C using ^1H MRS. The spectral information derived from *in vitro* ^1H MAS NMR studies was used to define the assignment and interpretation of ^1H MRS *in vivo*.

A cohort of 59 patients with biopsy-proven chronic hepatitis C was stratified by fibrosis stage and genotype. *In vivo* MR spectra were obtained using a Philips Achieva clinical MR system at 1.5T. MR spectra were obtained with and without water-suppression. MR spectra were analysed using prior knowledge informed by high-resolution *in vitro* studies of liver tissue from **Chapter 4**. Differences in lipid quantity and composition, according to fibrosis stage and genotype seen *in vitro* were validated in this separate *in vivo* cohort.

Conclusion: Lipid composition in chronic hepatitis C was assessed non-invasively using ^1H MRS, informed by high resolution NMR studies of intact tissue. Total lipid increased and polyunsaturation decreased with increasing fibrosis stage. This technique may provide insights into the pathogenesis of chronic liver diseases and could be added to other MR sequences in subsequent studies.

5.2 Introduction

Hepatic lipid composition is an important factor in the aetiology and pathogenesis of chronic liver disease (**section 1.5**). In the context of hepatitis C infection, hepatic steatosis is associated with more severe fibrosis, with more rapid progression of disease (Adinolfi *et al.*, 2001; Rubbia-Brandt *et al.*, 2004), while response to antiviral treatment has been associated with a decrease in steatosis in patients with genotype 3 (Reddy *et al.*, 2008). Hepatic steatosis in hepatitis C is thought to evolve in a genotype-related manner. Virologically-mediated steatosis is associated more with genotype 3 (Rubbia-Brandt *et al.*, 2000), mediated in part by viral inhibition of fatty acid export mechanisms (Hourieux *et al.*, 2007) and steatosis associated with the presence of insulin resistance is commonly found in non-genotype 3 (particularly genotype 1) disease (Asselah *et al.*, 2006; Camma *et al.*, 2006; Hui *et al.*, 2003; Moucari *et al.*, 2008).

The current gold-standard for the clinical assessment of hepatic lipid is liver biopsy, which provides a semi-quantitative assessment of hepatocellular lipid droplets, in addition to evidence of inflammation and fibrosis (Kleiner *et al.*, 2005). A number of validated scoring systems are used for the assessment of the severity of hepatitis C-related chronic liver disease, including the modified histological activity index of Ishak and the METAVIR scoring systems (Bedossa and Poynard, 1996; Ishak *et al.*, 1995; METAVIR, 1994). These systems assess liver disease severity in terms of fibrosis stage and inflammatory features. To this may be added an assessment of lipid load on the basis of the proportion of hepatocytes with visible lipid droplets. Recently, histological lesions associated with non-alcoholic steatohepatitis (NASH) were studied in patients with chronic hepatitis C in the context of clinico-biological correlates (Bedossa *et al.*, 2007). However, histological assessment of hepatic lipid does not provide information on lipid composition. Non-invasive techniques for the assessment of hepatic lipid composition are required.

Murine and human studies of intact liver tissue using high resolution ^1H MAS NMR spectroscopy have shown the ^1H MR spectrum to be dominated by water, lipid and choline

resonances (**section 2.6.3.1, Figure 3-2, Figure 4-1**). Moreover, characteristic metabolic perturbations associated with disease severity in patients with hepatitis C have been noted, and MR indices derived, in **Chapter 4**. In this study, the aim was to translate and validate these findings in a cohort of patients with hepatitis C studied *in vivo*.

5.2.1 Technical criticism of previous hepatic ^1H MRS studies

In vivo ^1H MRS has been shown to be a quantitative tool for the non-invasive assessment of hepatic total lipid load (Browning *et al.*, 2004; Szczepaniak *et al.*, 2005; Thomas *et al.*, 2005) and the techniques used have been discussed in **section 1.7.3.6.3**. Absolute quantification of intrahepatic lipid has been estimated by calibration of the number of MR-visible protons within the lipid resonances, with quantification of hepatic lipid extracts (Longo *et al.*, 1995). The relative quantification of hepatic lipid has been derived as a simple ratio to the hepatic MR spectral water resonance, although this method of quantification is not directly comparable to absolute concentrations of intrahepatic lipid (Thomas *et al.*, 2005).

Studies have used ^1H MRS to stratify chronic liver disease (Cho *et al.*, 2001; Orlacchio *et al.*, 2008), but the assignment of metabolites in such studies is controversial. In the study by Cho and colleagues, metabolites were likely to have been assigned to MR spectral noise (Lim *et al.*, 2003a). The study by Orlacchio and colleagues aimed to stratify patients with chronic hepatitis C according to disease severity. For the assignment of *in vivo* MR spectral resonances, they used a study of high resolution ^1H NMR spectroscopy of aqueous extract of liver tissue (Bell *et al.*, 1993), thus assigning an aqueous “glutamine-glutamate” complex to a large lipid resonance (Duarte *et al.*, 2005).

Recently, hepatic lipid content was assessed in subjects with differing degrees of hepatic steatosis, with a calculation of the degree of fatty acyl saturation, demonstrating the potential for ^1H MRS to assess lipid composition (Johnson *et al.*, 2008). Models of oils of known composition were studied at 1.5T and indices of fatty acid saturation derived. Patients with

differing degrees of hepatic steatosis were then studied using ^1H MRS at 1.5T. Scrutiny of the published MR spectra from the oils showed the chemical shift difference between the methylene resonance and the diallylic resonance was $\delta 1.5\text{ppm}$, consistent with results from *in vitro* MR studies of intact liver of $\delta 1.47\text{ppm}$ (Duarte *et al.*, 2005) and $\delta 1.48\text{ppm}$ (Martinez-Granados *et al.*, 2006). However, the relative chemical shift between the methylene resonance and the peak assigned as the “diallylic” resonance in humans was $\delta 1.9\text{ppm}$, more consistent with the published chemical shift difference between methylene and tCho resonances of $\delta 1.90\text{ppm}$ (Duarte *et al.*, 2005) and $\delta 1.88\text{ppm}$ (Martinez-Granados *et al.*, 2006). This criticism is supported by **Figure 3-1** in **Chapter 3**, showing high resolution ^1H MAS NMR spectra from normal and steatotic liver from mice aligned with a ^1H MAS MR spectrum from a sample of oil of known composition. Thus, in the paper by Johnson and colleagues, the significant differences were seen between groups should have been interpreted as being a result of a reduced tCho:lipid ratio, rather than a reduction in PUI (Cobbold *et al.*, 2008b).

These fundamental criticisms of previous work provide lessons for subsequent studies: the model or reference used for the assignment of resonances should be physiologically relevant to the samples being tested and the reference peak used to assign spectra should be present in both the model and the subject.

5.3 Hypotheses

The hypotheses for this study were that:

1. Information gained from high resolution ^1H MAS NMR spectroscopy studies of intact liver tissue would inform the assignment and analysis of lower resolution ^1H MR spectra *in vivo*
2. Indices of lipid composition observed *in vitro* can be reproduced in a separate cohort, *in vivo*

5.4 Aims

The aims of this study were:

1. To define prior knowledge for the analysis of ^1H MR spectra *in vivo* using high resolution ^1H MAS NMR spectral data
2. To validate the indices tested in **Chapter 4** in a separate cohort of patients *in vivo*.

5.5 Materials and methods

5.5.1 Recruitment

59 patients with chronic hepatitis C were prospectively recruited from Imperial College Healthcare Trust from September 2006 to January 2008. Ethical approval for the study was obtained prospectively from the regional ethics committee in accordance with the 1975 Declaration of Helsinki. Patients were included if they were aged 18-65, had evidence of replicating hepatitis C-virus infection on HCV RNA testing, and had been referred for percutaneous liver biopsy for clinical indications.

Patients were excluded if they consumed >20g of alcohol per day, if they were obese (with a BMI >30kg/m²) or diabetic. Patients were also excluded if they were taking antiviral therapy; were co-infected with HIV or hepatitis B; were currently taking intravenous drugs, antihypertensive or lipid-lowering medications; had ongoing illness or had evidence of hepatic decompensation, defined by any of the following: jaundice, ascites, or hepatic encephalopathy.

All patients underwent percutaneous liver biopsy by an experienced hepatologist using a 16-gauge Menghini needle, which was sent immediately for histology. Biopsy samples for histology were fixed in formalin and embedded in paraffin wax, prior to staining with haematoxylin and eosin. All samples were scored according to the Ishak modified histological activity index (Ishak *et al.*, 1995) and by NASH Clinical Research Network semi-quantitative scoring system for cellular morphology, inflammation and fibrosis by an experienced hepatopathologist (Kleiner *et al.*, 2005). The patients were divided into three groups on the basis of the Ishak fibrosis stages as follows: group 1, mild fibrosis, stages 1-2; group 2, moderate fibrosis, stages 3-4; group 3, cirrhosis, stages 5-6.

5.5.1.1 ¹H MRS studies

A Philips Achieva 1.5T whole-body system (Philips Medical Systems, Best, The Netherlands) with a 4-channel SENSE body coil and Q body coil was used. Routine T₁-weighted survey images were used for voxel placement. An 8cm³ (2 x 2 x 2cm) voxel was placed in the right lobe of the liver, avoiding large blood vessels or biliary structures. A single voxel PRESS (point-resolved spectroscopy) sequence was used without water suppression for the assessment of total lipid content, and with water suppression for assessment of lipid composition (TR 2s, TE 40ms). 16 blocks, each of eight acquisitions per breath-hold, were acquired. Patients fasted for at least 6hr prior to scanning.

5.5.2 *In vivo* MR spectral analysis

Spectra were processed using MRUI software, v3.0 (www.mrui.uab.es/mrui) (Naressi *et al.*, 2001). Analysis was performed on spectra derived from a single block of eight acquisitions, as summation of blocks reduced the spectral resolution because of respiratory motion. Following Fourier transformation, DC correction was applied, followed by apodisation (line-broadening) of 5Hz. Manual phasing was performed and the reference peak (methylene fatty acyl functional group) set at δ 1.3ppm. Prior knowledge applying “soft”¹ constraints to the curve-fitting parameters in terms of chemical shift, phase and linewidth (derived from *in vitro* data as detailed in **Table 5-1**) was applied to integrate the spectral peaks using AMARES (Hamilton *et al.*, 2003; Vanhamme *et al.*, 1997). An example of curve-fitting using jMRUI and the newly applied prior knowledge is shown in **Figure 5-1**. The investigator (the author) was blinded to the clinical and biochemical details pertaining to each spectrum at the time of spectral analysis. In order to estimate the reliability of curve-fitting, all spectra were re-analysed separately by an additional blinded investigator (J. Patel, BSc student).

¹ “Soft” constraints refer to a specified range of values for a parameter. The curve is fitted optimally, within the range of values supplied as “prior knowledge”.

Table 5-1. Prior knowledge used for the analysis of *in vivo* ¹H MRS

Metabolite	Functional group	Chemical Shift <i>in vitro</i> (ppm)	Chemical shift <i>in vivo</i> starting values (ppm)	Constraints to chemical shift <i>in vivo</i> (ppm)	Linewidth constraints (Hz)	Phase constraints (°)
NWS						
Methyl fatty acyl group	-CH ₃	0.9	0.9	0.7-1.0	5-20	nil
Methylene fatty acyl group	-CH ₂ -	1.3	1.3	1.2-1.4	5-30	nil
Water	H ₂ O		4.7	4.5-4.9	5-40	nil
WS						
Methyl	-CH ₃	0.9	0.9	0.7-1.0	5-20	-20 to +20
Methylene	-CH ₂ -	1.3	1.3	1.2-1.4	5-30	-20 to +20
Methylene to α-methylene to Carboxyl	-CH ₂ -CH ₂ - COO	1.6	1.6	1.5-1.7	5-20	-20 to +20
Allylic	-CH ₂ - CH=CH-	2.04	2.0	2.0-2.1	5-20	-20 to +20
α-methylene to carboxyl	COO-CH ₂ - CH ₂ -	2.26	2.2	2.2-2.3	5-20	-20 to +20
Diallylic	=CH-CH ₂ - CH=	2.80	2.8	2.7-2.9	5-20	-20 to +20
Total Choline	-N(CH ₃) ₃	3.20-3.26	3.2	3.15-3.35	5-20	-20 to +20
Water residual	H ₂ O		4.7	4.5-4.9	5-30	-5 to +5
n-ene group	-CH=CH-	5.33	5.3	5.2-5.4	5-20	-20 to +20

Abbreviations: NWS, non water-suppressed; WS, water-suppressed

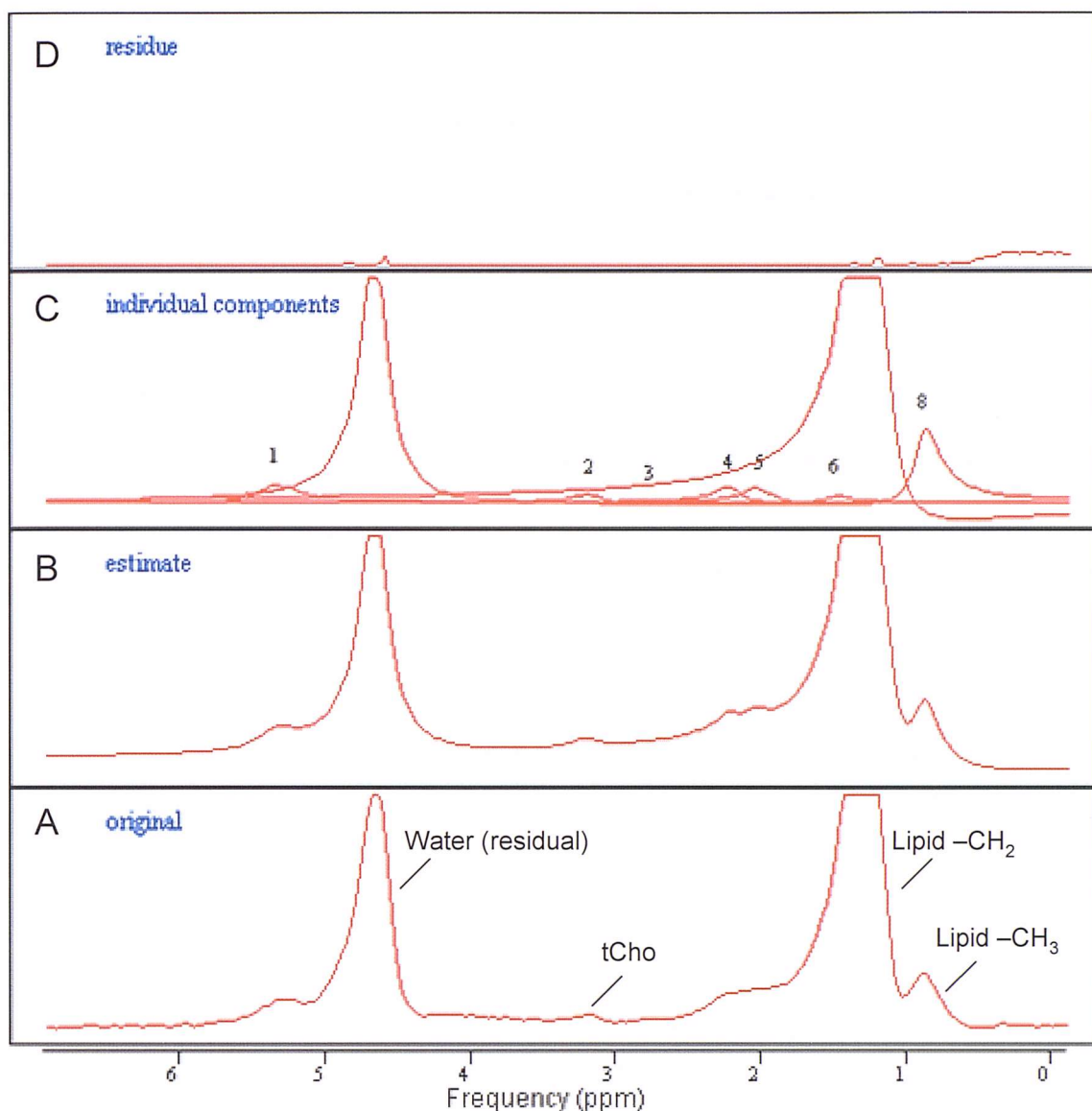


Figure 5-1. An example of the curve-fitting output using jMRUI.

The panels have been expanded vertically to show the smaller spectral resonances, leading to truncation of the display of the residual water and lipid -CH₂ peaks. Panel **A** shows the original water-suppressed spectrum, with major resonances labelled (tCho, total choline). Panel **B** shows the spectrum reconstructed from the curves specified by the prior knowledge supplied. Panel **C** shows the individual curves, as fitted. The numbers refer to the order that the curves were fitted. Panel **D** shows the residual, that is, the parts of the spectrum not fitted by the prior knowledge curve algorithm. A well-fitted spectrum should have little residual.

5.5.3 Statistical methods

SPSS v16 (SPSS Inc. Chicago, USA) was used for analysis of both cohorts with advice from a biomedical statistician. Clinical and biological indices were expressed as median values with interquartile range (or range). MRS-derived indices were compared using the Mann-Whitney U test for pair-wise comparisons. Correlation was performed using the Spearman rho test, with fibrosis stage (0-6) approximating a linear variable. Reliability assessment between investigators was assessed using the intraclass correlation coefficient (ICC). Correction for multiple comparisons was not performed as indices were correlated.

5.6 Results

5.6.1 Patient characteristics

The demographic and clinical characteristics of the 59 patients in this cohort are summarised in Table 5-2 and Table 5-3.

Table 5-2. Numbers of patients stratified by race and genotype.

Race	Genotype				Total
	1	2	3	4	
Europoid (British)	17	3	8	0	28
Europoid (non-British)	11	0	2	3	16
South Asian	1	0	3	0	4
Middle- Eastern	0	0	0	4	4
Black African	1	1	0	0	2
East Asian	0	1	1	0	2
Total	28	5	14	7	56

Patient numbers in the cohort of 59 patients studied using ¹H MRS are tabulated by genotype and race. Genotyping was unavailable for three patients. The Middle-Eastern group included those of Arab East African origin.

Table 5-3. Patient characteristics

	Total	Fibrosis group			Genotype		
		1	2	3	1	3	Other (2,4)
Number	59	27	21	11	30	14	12 #
Sex M/F	38/21	17/10	15/6	6/5	18/12	8/6	9/3
Age (years)	50 (15)	50 (18)	48 (10)	48 (14)	49.5 (14)	46.5 (10)	54 (20)
Genotype (no. 1/3)	30/14	15/4	9/7	6/3	N/A	N/A	N/A
Bx length, mm (range)	25 (10-49)	27 (10-48)	21 (11-49)	25.5 (10-47)	23.5 (10-48)	30 (11-49)	25 (10-38)
BMI	24.5 (5.09)	23.0 (4.44)	25.3 (7.82)	26.4 (4.46)	23.8 (4.45)	23.0 (7.25)	26.5 (5.5)
ALT, iu/L [0-40]	90 (95)	52 (89)	98 (73)	137 (100)	90 (65)	140 (85)	79 (101)
AST, iu/L [0-40]	60 (58)	39 (35)	79 (45)	106 (63)	59 (50)	90 (79)	58 (49)
Platelets, x10⁹/L [130-450]	209 (84)	214 (76)	186 (117)	181 (104)	213 (105)	197 (72)	202 (35)
Total Cholesterol, mmol/L [<6.5]	4.30 (1.36)	4.50 (1.17)	4.68 (1.35)	3.64 (1.12)	4.54 (1.12)	4.28 (1.83)	4.18 (1.06)
Triglycerides, mmol/L [<2.5]	0.96 (0.55)	1.00 (0.67)	1.02 (0.48)	1.58 (0.51)	1.03 (0.52)	0.94 (0.62)	0.94 (0.56)
HOMA-IR	1.97 (2.02)	1.78 (1.94)	2.16 (2.24)	2.26 (1.95)	1.69 (0.96)	2.26 (1.77)	3.09 (2.17)

Figures are median (interquartile range) unless otherwise specified. M, male; F, female; Bx, biopsy; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase. # genotype data missing for 3 patients. Values in bold are significant differences at the p=0.05 level using the Kruskal-Wallis test.

5.6.2 MR characterisation

Representative water-suppressed ^1H MR spectra *in vitro* and *in vivo* are shown in **Figure 5-2**, illustrating a number of specific lipid resonances including fatty acid functional groups. The total choline region ($\delta 3.18$ - $\delta 3.28$ ppm) (tCho) contains peaks representing choline, phosphocholine, glycerophosphocholine and phosphatidylcholine, with contributions from trimethylamine-N-oxide (TMAO) and betaine.

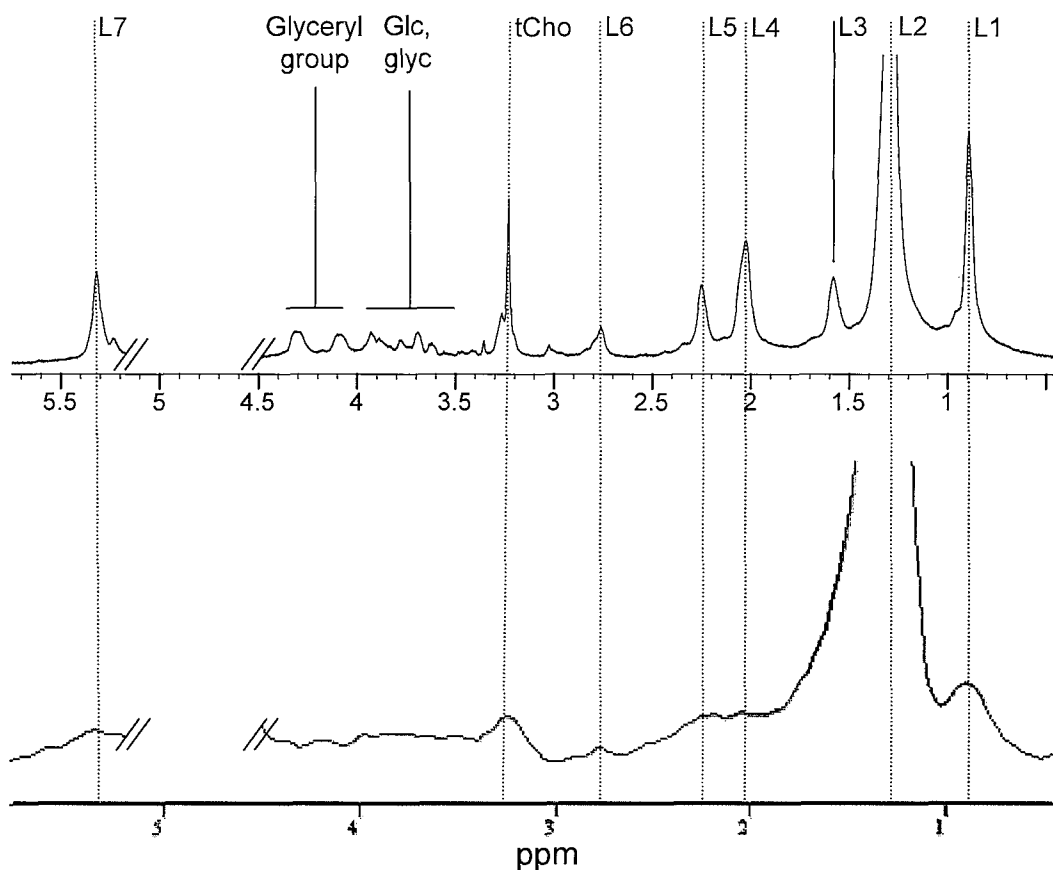


Figure 5-2. *In vitro* and *in vivo* MR spectra aligned by chemical shift.

The residual water resonance at $\delta 4.8$ - 5.2 ppm was removed as indicated. Key: L1, lipid CH_3CH_2 (methyl); L2 lipid $(\text{CH}_2)_n$ (methylene); L3 lipid $\text{CH}_2\text{CH}_2\text{CO}$ (methylene to L5); L4 lipid $\text{CH}=\text{CHCH}_2\text{CH}_2$ (allylic); L5 lipid $\text{CH}_2\text{CH}_2\text{CO}$ (α -methylene to carboxyl); L6 lipid $\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}$ (diallylic - "PUFA"); L7 lipid $\text{CH}=\text{CH}$ (*n*-ene - "MUFA"); tCho, total choline region with contributions from choline, phosphocholine, glycerophosphocholine, trimethyl amine oxide (TMAO) and betaine; glc, glucose; glyc, glycogen.

5.6.3 Peak assignment and fitting

Peak assignment *in vivo* was performed using higher resolution *in vitro* MR spectra, plotted along the same scale of chemical shift and both referenced to the dominant lipid peak, the methyl fatty acid resonance (**Figure 5-2**). By direct comparison to *in vitro* NMR spectra, only *in vivo* peaks corresponding to substantial peaks *in vitro* were assigned, reducing the chance of inappropriate peak assignment.

The spectra shape, relative size and position of peaks identified *in vitro* enabled appropriate prior knowledge for the jMRUI AMARES curve-fitting algorithm. Peak chemical shift, phase, linewidth and lineshape were subject to soft constraints as detailed in **Table 5-1**. Application of this prior knowledge to all *in vivo* MR spectra and blinding of the analyst ensured consistent unbiased analysis throughout.

The reliability of curve fitting, assessed independently by two blinded investigators, is displayed in **Table 5-4**. The MR spectra including water were fitted with high reliability. In the water-suppressed spectra, the “*n*-ene” (-CH=CH-) lipid peak was fitted by both investigators in 95% of cases, while the diallylic lipid peak was fitted in 77% of cases with high reliability (0.86). Thus, PUI values were available for 45 of the 59 patients in this cohort. The other peaks were fitted in 100% of cases.

Table 5-4. Reliability of curve fitting

Peak	ICC	95% CI	Fitted peaks (%)
<i>NWS</i>			
Water	0.982	0.968-0.990	100
Methylene	0.980	0.965-0.989	100
Methyl	0.776	0.604-0.874	96
<i>WS</i>			
" <i>n</i> -ene"	0.887	0.797-0.937	95
tCho	0.873	0.782-0.926	100
Diallylic	0.862	0.714-0.934	77
α -methylene to carboxyl	0.848	0.739-0.911	100
Allylic	0.774	0.610-0.869	100
Methylene	0.999	0.998-0.999	100
Methyl	0.942	0.900-0.966	100

Curve-fitting was performed by two independent observers. Abbreviations: NWS, non water-suppressed; WS, water-suppressed; tCho, total choline; ICC, intraclass correlation coefficient; CI, confidence interval.

5.6.4 Analysis of lipid composition

The MRS trends observed *in vitro* (**Chapter 4**) were confirmed in this separate *in vivo* cohort as shown in **Figure 5-3**: total lipid increased with increasing fibrosis ($r=0.48$, $p<0.0005$); SI correlated positively ($r=0.39$, $p=0.003$); the choline to lipid ratio was negatively correlated with fibrosis stage ($r=-0.52$, $p<0.0005$) and the PUI also decreased with increasing fibrosis ($r=-0.55$, $p<0.0005$).

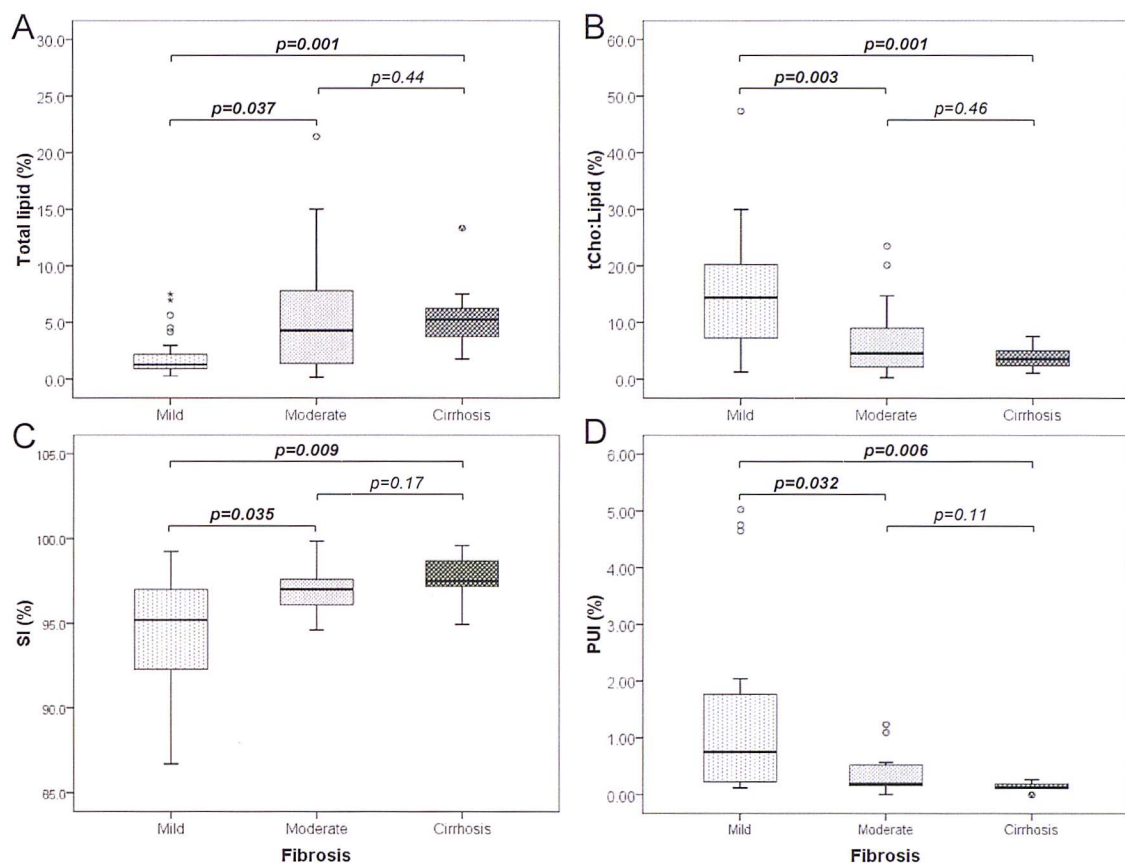


Figure 5-3. Indices validated at 1.5T plotted against fibrosis stage.

Categories are mild fibrosis (F1-2), moderate fibrosis (F3-4) and cirrhosis (F5-6). “Box and whisker” plots represent the median (horizontal line) and interquartile range (box) with the range of values up to 1.5 times the IQR within the range of each of the bars. Outliers are plotted individually beyond the bars. Significant *p*-values from the Mann-Whitney U test are displayed in bold, non-significant differences in normal weighted text. **A**, total lipid increased with severity of fibrosis, from 1.30% (1.49) through 4.30% (7.18) to 5.25% (2.93); **B**, the total choline to lipid ratio decreased from 14.43% (13.66) through 4.57% (7.89) to 3.58% (3.32); **C**, the saturation index increased from 95.21% (5.73) through 97.02% (1.64) to 97.52% (1.85); **D**, the polyunsaturation index decreased from 0.75% (1.70) through 0.19% (0.39) to 0.13% (0.12).

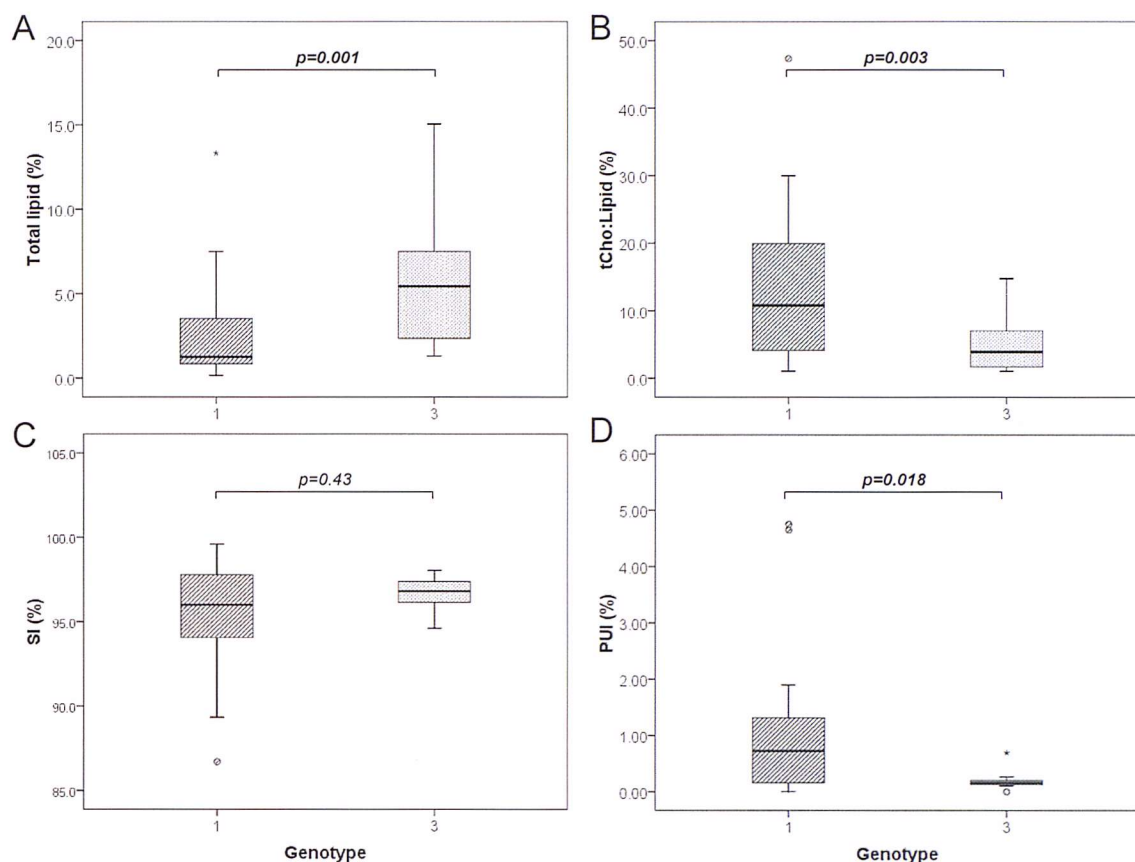


Figure 5-4. Indices validated at 1.5T plotted by genotypes 1 and 3.

“Box and whisker” plots represent the median (horizontal line) and interquartile range (box) with the range of values up to 1.5 times the IQR within the range of each of the bars. Outliers are plotted individually beyond the bars. Significant p-values from the Mann-Whitney U test are displayed in bold, non-significant differences in normal weighted text. **A**, total lipid was lower in genotype 1, 1.26% (2.95) than genotype 3, 5.45% (5.50); **B**, the total choline to lipid ratio was higher in genotype 1, 10.8% (16.0) than genotype 3, 3.94% (5.46); **C**, the saturation index was 96.0% (3.83) for genotype 1 and 96.8% (1.33) for genotype 3; **D**, the polyunsaturation index was higher in genotype 1, 0.73% (1.22) than genotype 3, 0.16% (0.09).

Differences in indices according to genotype are shown in **Figure 5-4**, and confirm those seen *in vitro* (**Figure 4-3**). However, while the absolute measures of total lipid were similar between groups, the absolute total choline and (poly)unsaturation indices were lower in the *in vivo* cohort compared to the *in vitro* cohort (**Chapter 4**). To confirm the observation *in vitro* that the total lipid measurement increased with increasing severity of disease in genotype 1, but not in genotype 3, total lipid was plotted against fibrosis severity and stratified by genotype (**Figure**

5-5). Indeed, in genotype 1, total lipid correlated with severity of fibrosis ($r=0.46$, $p=0.016$), but in genotype 3, there was no correlation with disease severity ($r=-0.04$, $p=0.91$). This discrepancy was most notable in those with mild disease.

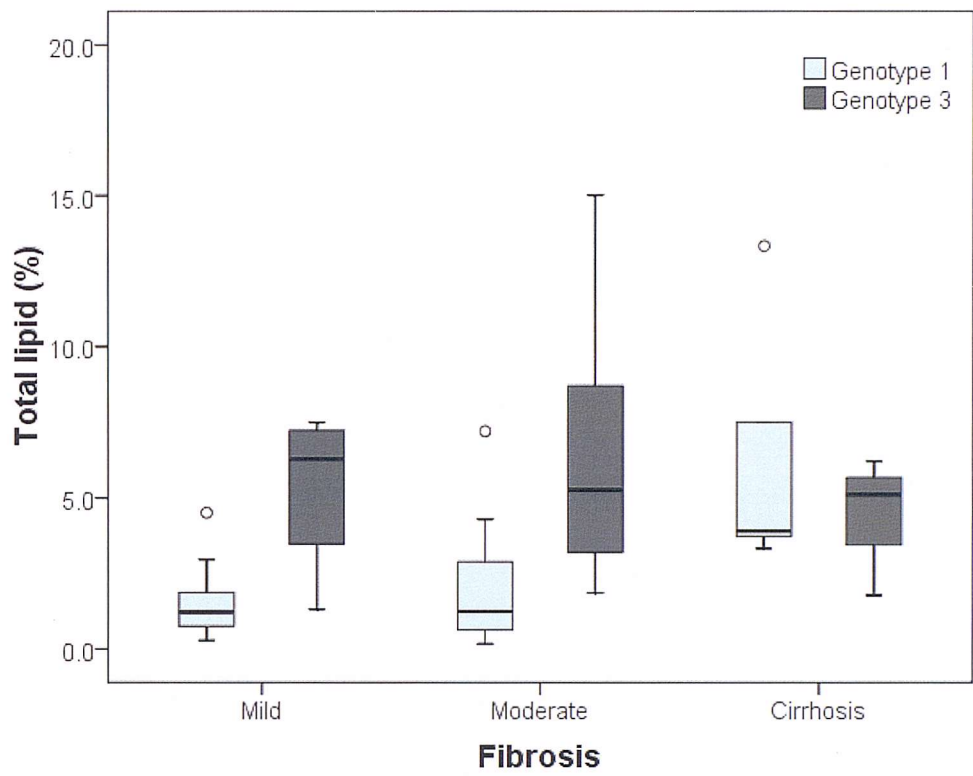


Figure 5-5. Percentage total lipid signal plotted by fibrosis, stratified by genotype.

“Box and whisker” plots represent the median (horizontal line) and interquartile range (box) with the range of values up to 1.5 times the IQR within the range of each of the bars. Outliers are plotted individually beyond the bars.

5.7 Discussion

The interpretation of *in vivo* ^1H MR spectra at 1.5T was aided by information obtained from the high resolution ^1H MAS NMR spectra from liver biopsy tissue. Indices tested in **Chapter 4** were validated here in the separate *in vivo* cohort. The *in vivo* cohort was similar to the *in vitro* cohort in terms of distribution of patient characteristics, supporting the comparison of MR indices. Total lipid and the degree of fatty acid saturation increased with increasing disease severity, and in genotype 3 compared with genotype 1. The extent of fatty acid polyunsaturation decreased independently with increased fibrosis and with increasing steatosis. The biological significance of these indices and differences has been discussed in **section 4.5.1**.

The importance of using relevant *in vitro* data to inform interpretation of *in vivo* MR spectra is reinforced by the errors seen in other studies and described in **section 5.2.1**. When using low resolution MR spectra, where information is obtained at the limit of the sensitivity of the technique, such mistakes are understandable, but would have been avoided by using this approach. The use of oils as a model may be useful to calibrate equipment, but cannot be considered to be biologically representative. Therefore, the results presented here represent a proof-of-principle for the non-invasive analysis of lipid composition using ^1H MRS.

HOMA-IR scores were variable within histological groups, but correlated weakly with increasing fibrosis stage ($r=0.29$, $p=0.03$) and with total lipid ($r=0.32$, $p=0.02$), consistent with recent studies assessing the effect of insulin resistance, steatosis and fibrosis in hepatitis C, which showed that HOMA-IR was a significant predictor of fibrosis stage (Cua *et al.*, 2008; Hui *et al.*, 2003). In this cohort, the median HOMA-IR was higher in patients with genotype 3 than those with genotype 1 (although non-significantly), contrary to those larger studies. This current study was not designed to look at differences in insulin resistance and genotype, so is underpowered to make such conclusions. Race may be a confounding factor in such analyses as the prevalence of insulin resistance and high levels of visceral adipose tissue is known to vary accordingly. For example, there is a high prevalence of insulin resistance, central obesity,

diabetes and coronary heart disease in those of South Asian origin (Despres *et al.*, 2000; Tillin *et al.*, 2005), while there is also a relatively high prevalence of genotype 3 HCV in the Indian subcontinent (Fang *et al.*, 1997). It might be hypothesized, then, that in a cohort of patients where a substantial number originate from South Asia, the background risk of T2DM and insulin resistance might confound the genotype-related effects on IR and intrahepatic lipid. Such an analysis was not possible with the relatively small number of patients studied in this cohort, particularly as obesity and diabetes were exclusion criteria for this study. Future studies aiming to assess intrahepatic lipid composition in patients with hepatitis C should control for race and, ideally, diet.

Other *in vivo* ^1H MRS studies have demonstrated non-invasive estimation of total hepatic lipid (Longo *et al.*, 1995; Szczepaniak *et al.*, 2005). Reproducibility analysis showed a coefficient of variation for the quantification of hepatic triglyceride of 8.5% (Szczepaniak *et al.*, 2005), but these studies did not assess lipid composition or the presence of other metabolites. Studies of *in vivo* ^1H MRS in chronic liver disease have demonstrated spectral differences between patients with disease of differing severity (Cho *et al.*, 2001; Orlacchio *et al.*, 2008). Additionally, Orlacchio and colleagues demonstrated an increase in the dominant lipid resonances with increasing disease severity in 30 patients with chronic hepatitis C (Orlacchio *et al.*, 2008). However, in the current study, the assignment of spectral resonances was informed by physiologically relevant models, ensuring appropriate assignment of resonances and preventing the assignment of metabolites to spectral noise (Lim *et al.*, 2003a).

5.7.1 Limitations and potential solutions

Some limitations are inherent to the technique of ^1H MRS. In particular, sensitivity is low and the output is the relative amount of MR-visible molecular functional groups, rather than a molar concentration of metabolites within a sample. Moreover, the length of fatty acid chains or the individual fatty acid species are not directly identifiable or quantifiable.

5.7.1.1 *Sampling variability*

A voxel or region of interest was used to obtain MRS data *in vivo*. If pathology is not evenly distributed, there may be a degree of sampling variability. There is a trade-off between the use of a large or small voxels. When using a large voxel, there is less sampling variability, greater signal, but poorer resolution on account of magnetic field gradients and inhomogeneity artifact. When using a small voxel, less signal is produced, the risk of sampling variability is greater, but resolution is improved. On the basis of preceding work in our group, an 8cm³ voxel was considered to be the appropriate compromise for these studies (M. Wylezinska, physicist, personal communication). Using an 8cm³ voxel *in vivo*, the portion of liver tested is around 1000x greater than that sampled by biopsy. Sampling variability of ¹H MRS *in vivo* is therefore minimal compared to the reference standard, liver biopsy. However, in the future, placement of more than one voxel (for example within both the right and left lobes of the liver) would enable spatial variability of spectral differences to be assessed.

5.7.1.2 *Delay from biopsy to investigation*

There was a delay between liver biopsy and *in vivo* MRS studies of up to one year. In this study, biopsy was used as a classification tool rather than as a strict reference standard, as histological assessment of liver biopsy was not used to assess the lipid profile. As the fibrosis stage is unlikely to change within a year, the effect of such a delay on the classification is likely to be insignificant (Marcellin *et al.*, 2002; Poynard *et al.*, 1997). All other clinical and biochemical parameters were obtained on the day of MR investigation.

5.7.1.3 *Limitations of spectral quality and analysis*

The resolution and SNR of the *in vivo* spectra at 1.5T limited the peaks that could be reliably estimated. The intraclass correlation of main resonances showed that the larger lipid peaks were fitted with excellent reliability between raters, but that the PUFA peak was fitted by both analysers in 77% of cases, although when fitted, the reliability was good. The values of the smaller resonances, such as the allylic and diallylic peaks *in vivo* were lower than those assessed *in vitro*. The lower SNR *in vivo*, together with the broader linewidths, is likely to have led to

underestimation of these resonances *in vivo* compared to the integrals observed *in vitro*. Accordingly, while estimation of total lipid may be quantified using MRS (Szczepaniak *et al.*, 2005), assessment of lipid composition may currently be considered quantitative only relative to other subjects studied on the same system or longitudinally. A direct, within-patient correlation from NMR of the biopsy sample *in vitro* to hepatic MRS *in vivo* would be useful to quantify the relationship between the resonances found in each setting in order to try to identify a conversion constant. Such an analysis was not performed as insufficient numbers of subjects for whom biopsy tissue had been taken for analysis were recruited for *in vivo* MR scanning.

Suppression of the water resonance *in vivo* was variable, in part due to variable patient motion. As the “*n*-ene” --CH=CH-- functional group resonance at $\delta 5.3\text{ppm}$ lies close to the residual water peak, there was a risk of variable constructive interference between the peaks. Accordingly, this resonance was excluded from incorporation into lipid indices (Johnson *et al.*, 2008).

MRS performed on systems at higher field strength (such as 3T) and better control of motion artifact will enhance spectral resolution and the SNR, and enable more reliable fitting of further resonances (Fischbach and Bruhn, 2008), but the need for physiologically-relevant assignment of peaks remains important. In this study, the investigation of resonances in intact tissue *in vitro* will provide a reference for future *in vivo* MRS studies. There is considerable interest in the relaxation properties (such as T_2) of metabolites in different tissues and differing disease states (Fischbach and Bruhn, 2008). Such effects should be studied directly in future studies. By collecting data at two or more echo times, any systematic difference in T_2 according to disease severity would be detected by a difference in the signal reduction in a resonance with increasing echo time between samples of differing disease severity. A direct calculation of the T_2^* (measured T_2) could be calculated by using a STEAM (stimulated echo acquisition mode) sequence of multiple echo times in health and disease. Although T_2 times may lead to MR spectral differences *in vivo*, the *in vitro* pulse and collect fully-relaxed NMR spectra were independent of T_2 . As the trends were similar in both cohorts, it may be surmised that the effect

of T_2 on the *in vivo* MR spectral integrals is likely to be small compared to the effect of disease severity *per se*.

5.8 Conclusions

To conclude, lipid composition in patients with chronic hepatitis C has been assessed non-invasively at 1.5T. An increase in total lipid and a decrease in lipid polyunsaturation have been observed with increasing hepatic fibrosis, while increased intrahepatic lipid was observed non-invasively in patients with genotype 3. The non-invasive assessment of lipid composition in chronic liver diseases using ^1H MRS adds a biologically and clinically relevant dimension to current disease assessment and the technique has potential to provide signs of efficacy in longitudinal studies in response to therapies which modulate hepatic lipid.

6 NON-INVASIVE ASSESSMENT OF HEPATITIS C-RELATED CHRONIC LIVER DISEASE

6.1 Abstract

The development of effective non-invasive biomarkers to assess the severity of chronic liver disease is of importance both in clinical practice and for the development of novel antifibrotic therapies. Combinations of markers may improve the ability to discriminate disease severity. The aim of this study was to compare four validated serum and ultrasound-based non-invasive markers of hepatic disease severity head-to-head: AST to platelet ratio index (APRI); Enhanced Liver Fibrosis (ELF) panel; transient elastography (TE) and ultrasound microbubble hepatic transit times (HTT). It was hypothesised that combining the markers in steps according to increasing cost and complexity would enable the relative contributions of each marker to be assessed. Regression analysis was performed to predict fibrosis stage and necro-inflammatory score in three-stage models and to establish the contribution of each marker.

Sixty seven patients with biopsy-proven chronic hepatitis C were recruited prospectively into the study. ELF and TE predicted cirrhosis with a diagnostic accuracy of 91% and 90%, respectively, and mild from more severe disease with a diagnostic accuracy of 82% each. APRI predicted cirrhosis and mild disease with a diagnostic accuracy of 86% and 83% respectively, while HTT performed least well. Either ELF or TE combined with APRI significantly enhanced the prediction of fibrosis stage, but all three combined did not improve the model further.

Conclusion: a combination of APRI with either ELF or TE effectively predicted fibrosis stage using a three-group model of fibrosis, but combinations of three or more tests led to redundancy of information. HTT did not enhance the combinations of other markers for the prediction of fibrosis. Large-scale cost-benefit analyses are required to evaluate further the additional benefit of marker combinations.

6.2 Introduction

The development of effective biomarkers of the severity of chronic liver disease is of importance, both in clinical practice and for the development of novel antifibrotic therapies. The currently accepted reference standard, histological examination of liver biopsy, is hampered by sampling variability, the semi-quantitative nature of scoring systems, inter-observer variability and, importantly, the risk of discomfort, significant morbidity and a small but significant risk of death to the patient, as detailed in **section 1.6.4**. Patients with CHC are an appropriate cohort for the investigation of markers of chronic disease severity (**section 1.4**).

The field of biomarker development for the assessment of disease severity and fibrosis in chronic liver disease is burgeoning and broadly comprises the development of serum markers, singly or in combination as *serum panel markers* and imaging-based technologies, including ultrasound and magnetic resonance techniques (**section 1.7**).

Although fibrosis itself is a continuous process and histological scoring systems divide fibrosis into five or seven stages, statistical analysis of disease severity has relied on two (histological) class (“binary”) analysis in many studies (such as cirrhosis or absence of cirrhosis, significant fibrosis or insignificant fibrosis), based on the commonly-expressed receiver operating characteristic (ROC) curve. Such analyses are expressed in readily understandable forms, such as sensitivity, specificity, positive predictive values and negative predictive values. These figures depend on the prevalence of the disease state in the population studied and, correspondingly, may be skewed by selection bias (Poynard *et al.*, 2007). It is often important to distinguish between more than two histological groups to determine where an individual exists within a range of disease severity.

Markers of varying cost and complexity are being developed with evidence of accuracy in a number of studies. However, for such markers to be adopted clinically, they should be cost-effective, relatively easy to perform and acceptable to the patient. Combinations of serum

markers, including APRI, FibroTest and Forns Index (Leroy *et al.*, 2007; Sebastiani *et al.*, 2006) and serum markers (APRI and FibroTest) with transient elastography have been studied (Castera *et al.*, 2005). A combination of imaging-based and serum markers may provide the best assessment of liver disease severity, as the differing modalities assess different parameters, which in combination may reduce redundancy between tests.

The APRI score is a simple derivative of routine blood markers: AST and the platelet count, validated in a number of chronic liver disease settings (Wai *et al.*, 2003; Wai *et al.*, 2006). It is easily calculated at the bedside and available globally. The Enhanced Liver Fibrosis (ELF) panel is a proprietary algorithm of extracellular matrix components (tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), hyaluronic acid (HA) and the aminoterminal peptide of procollagenase 3 (PIIINP)), which estimates fibrosis on the basis of intermediates of fibrogenesis (Rosenberg *et al.*, 2004). Transient elastography (TE) is a marker of liver stiffness, which correlates with chronic liver disease severity, in terms of fibrosis stage (Castera *et al.*, 2005; Sandrin *et al.*, 2003; Ziol *et al.*, 2005), portal pressures (Kazemi *et al.*, 2006; Vizzutti *et al.*, 2007) and inflammation (Coco *et al.*, 2007) in a number of disease settings (Corpechot *et al.*, 2006; Ganne-Carrie *et al.*, 2006) (**section 1.7.3.4**). Dynamic contrast-enhanced ultrasound to derive HTT has been shown to stratify chronic liver disease in patients with hepatitis C (Lim *et al.*, 2005b; Lim *et al.*, 2006; Ridolfi *et al.*, 2007) and in comparison to Child-Pugh score (Blomley *et al.*, 2003). The estimated costs for the use of these markers are tabulated in **Table 6-1**, with a summary of the resources required to provide the service.

Neither the ELF panel nor HTT has been compared head-to-head to other non-invasive markers in the setting of chronic hepatitis C. The difference in diagnostic performance of markers has not been clearly demarcated, with many markers performing similarly within and between studies. Accordingly, a pragmatic step-wise approach to employing such markers is to start with the cheaper, more widely-available, easy-to-perform tests, which are least inconvenient to the patient, then to add in further markers according to additional benefit, availability, cost and patient acceptability, as outlined in **Table 6-1**.

Table 6-1. Comparison of marker costs and complexity.

Marker	Cost / event	Capital outlay	Personel	Notes
APRI	< £1	No	Phlebotomy	Routine laboratory tests Globally available
ELF	c. £180^a	No	Phlebotomy and transport	1 UK-based laboratory, cost subject to contract
TE	c. £200 ^b	Yes £54 000 ^c	Single operator , simple training	Real-time results. Time taken: c. 5min Increasing availability globally
HTT	c. £60 ^d	Yes c. £100 000 ^e - £200 000	Two operators required. One sonographer with specialist training One assistant, for cannulation and contrast injection	Real-time results. Time taken: c. 20min May be performed as part of a routine abdominal ultrasound procedure Performed in a few specialist centres (UK, France, Australia, Japan)

Costs are not directly comparable and do not include economies of scale. These figures are produced to support the combinations of markers used in this study and cannot be used as the basis for service or research costings.

^a ELF cost on “per sample” basis inclusive of VAT (September 2008). Costs may differ according to number of samples and potential discounts to the NHS and for academic studies.

^bTE using FibroScan[®] quoted by the Liver Unit at St Mary’s Hospital, Imperial College Healthcare Trust for a single procedure, inclusive of staffing time and overheads.

^cPurchase cost for FibroScan[®] in the UK, from Artemis Medical Ltd, quoted September 2008 at an exchange rate of £1 to €1.2, exclusive of VAT, inclusive of staff training, warranty and 1 year service contract.

^dCost of 1 vial of Sonovue to the Department of Radiology, Hammersmith Hospital, Imperial College Healthcare Trust, September 2008. Excludes staff and room time, hardware and interpretation of results.

^eEstimated cost of Toshiba Aplio ultrasound machine from October 2005. The cost of ultrasound equipment varies widely. HTT requires “vascular recognition imaging” or similar software to be supported on the system used.

6.3 Aims

The aims of this study were:

- a. To evaluate the diagnostic performance and reliability of APRI, ELF, TE and HTT head-to-head, compared to liver biopsy.
- b. To employ a statistical model of three histological groups for the assessment of accuracy.
- c. To assess the performance of these markers combined in a stepwise manner, based on cost and practicality of tests, and their individual contribution to each model.

6.4 Methods

Ethical approval for the study was obtained from the regional ethics committee and the study was conducted in accordance with the Declaration of Helsinki. 67 patients with chronic hepatitis C were recruited prospectively from the Liver Unit at Imperial College Healthcare Trust from March 2006 to January 2008. All gave written, informed consent to participation in the study. Patients were included if they were aged 18-65 years, had undergone liver biopsy within one year of investigation and if they had evidence of ongoing viral replication on hepatitis C RNA testing. Patients were excluded if they consumed >20g of alcohol per day on average, if they were taking antiviral therapy, if they were co-infected with HIV or hepatitis B, or if they were currently taking intravenous drugs or antihypertensive medications. Patients were also excluded if they had renal impairment, evidence of ischaemic heart disease, congestive heart failure, malignancy, autoimmune, inflammatory or infectious illness including, but not limited to, rheumatoid arthritis, inflammatory bowel disease, systemic sclerosis, diabetes mellitus, hyperthyroidism and tuberculosis. Patients were excluded if they had evidence of hepatic decompensation, defined by any of the following: jaundice, ascites, hepatic encephalopathy, or evidence of oesophageal or gastric varices on endoscopic examination.

To assess the reliability of the markers, 26 patients attended for investigation on a second occasion, 7 days after the first investigation point. All 26 patients underwent all tests successfully on both occasions.

6.4.1 Histology

Percutaneous liver biopsy was obtained from all participants in the clinical setting and all samples were reassessed by a single, blinded hepatic histopathologist according to the modified histological activity index (Ishak *et al.*, 1995). In this scoring system, fibrosis was assessed on a seven-point scale (0-6), with stages 5 and 6 considered to have cirrhosis. Disease activity or

necro-inflammation (inflammation) was assessed on a 19-point scale (0-18). Patients were excluded if their biopsy was shorter than 10mm.

All patients underwent haematological, biochemical and virological investigations on the day of their participation in the study. An abdominal ultrasound scan was performed on all subjects to exclude biliary obstruction, presence of space-occupying lesions and to confirm normal hepatobiliary anatomy.

6.4.2 Non-invasive markers

All blood samples and imaging tests were obtained on the same visit and within 2hr of each other. Patients fasted overnight or for a minimum of 6hr prior to the investigations to reduce variation in hepatic and splanchnic flow, which could be attributed to digestion.

6.4.2.1 *APRI*

The APRI, as defined in **Equation 6-A**, was derived from routine clinical blood tests obtained at venepuncture (Wai *et al.*, 2003). Each part of the index is divided by the upper limit of normal (ULN) for the measure in a particular laboratory.

Equation 6-A

$$\text{APRI} = \frac{100 (\text{AST} / \text{AST [ULN]})}{\text{Platelet count (x10}^9\text{)}}$$

6.4.2.2 *ELF*

Serum samples were obtained on the same occasion as other blood tests at venepuncture. Samples were collected in serum separator tubes and centrifuged at 3000rpm for 10 minutes, then stored at -80°C according to company instructions prior to processing by IQur Ltd (IQur, Southampton, UK). The output consisted of a numerical continuous variable for each sample.

6.4.2.3 *Liver stiffness measurement*

Liver stiffness measurement was obtained by TE using FibroScan® (EchoSens, Paris, France) as previously described (Ziol *et al.*, 2005). The operators were formally trained by the manufacturers and had performed at least 50 examinations by the start of the study. TE was considered valid if 10 measurements were obtained with a success rate of >60% and an interquartile range of values <30% of the median value. Patients with invalid scores were excluded from the head-to-head analysis.

6.4.2.4 *Hepatic transit times*

HTTs were performed using the microbubble contrast agent, SonoVue® (Bracco, Milan, Italy), based on the methods of previous studies (Lim *et al.*, 2006; Searle *et al.*, 2008). Briefly, a region of interest was selected containing hepatic artery and either the right or middle hepatic vein. A scanner-specific contrast agent software, vascular recognition imaging (Toshiba Medical Systems Europe, Zoetermeer, Netherlands) using ultralow acoustic power (mechanical index of 0.6) was used. A 21-gauge cannula with a three-way tap was inserted into an antecubital fossa vein. The microbubble contrast agent was reconstituted in accordance with the manufacturer's instructions. A 1mL rapid bolus of Sonovue®, followed by a 8mL normal saline flush were administered, as described previously (Lim *et al.*, 2006). A timer was started at the beginning of the Sonovue® injection. The time of arrival of the contrast agent (defined as the first appearance of a definite increase in signal) in the hepatic artery (the hepatic artery arrival time) and then the hepatic vein (the hepatic vein arrival time) was recorded in real time by the operators, blinded to the patient details. The HTT was calculated as the hepatic artery arrival time subtracted from the hepatic vein arrival time. Clearance of the contrast agent from the circulation took approximately 5 minutes, after which the process was repeated. The recorded HTT was the mean of the two measures, in seconds.

6.4.3 Statistics

SPSS v16 (SPSS Inc. Chicago, USA.) was used for statistical modelling in collaboration with an experienced biomedical statistician (Dr Peter Colman, Pfizer UK Ltd., Sandwich, UK). Binary analysis was performed using receiver operator characteristic (ROC) curves, separating patients into two groups based on histological features (fibrosis stage or necro-inflammatory grade). The diagnostic accuracy was the area under the ROC (AUROC), expressed as a percentage. Cut-off values for each marker (or test) in the cohort were established where the sensitivity and specificity were maximal and corresponding positive and negative predictive values were calculated.

Reliability was assessed using the intraclass correlation coefficient (ICC). The ICC was used to indicate how much variation in the dataset was found between the patients (random) compared to the variation between the visits (fixed). An ICC of >0.8 indicates that variation is predominantly between patients, with good agreement between visits, indicating a reliable test. An ICC of <0.4 indicates that the variation is predominantly inherent in the test.

For the three-group analysis, a non-orthogonal, ordinal regression analysis was performed to identify the goodness-of-fit for each marker compared to the reference standard, histological assessment of liver biopsy (Norusis, 2008). This process was carried out to model marker performance against fibrosis stage and (necro)-inflammation. The fibrosis stage was divided into three groups: mild fibrosis (stages 0-2), moderate to severe fibrosis (stages 3-4) and cirrhosis (stages 5-6). Similarly, the necroinflammatory score was divided into mild (0-3), moderate (4-6) and severe inflammation (7+). The goodness-of-fit of the model was described by the deviance (chi-square) divided by the degrees of freedom, with a lower deviance denoting better fit for a given number of degrees of freedom. The accuracy of the models was denoted by the proportion of patients whose grouping was predicted correctly, and expressed as a percentage. The significance of the contribution of a marker to the model (combination) was

denoted by a p value and confidence intervals, with a p-value of <0.05 and confidence intervals not spanning zero denoting a significant contribution of that marker to the model.

Combinations of markers were constructed as shown in **Table 6-2** according to increasing cost and complexity as detailed in **Table 6-1**. The diagnostic accuracy and the significance of the relative contribution of each marker to the model were also assessed using the three-group ordinal regression analysis. The model was structured in a step-wise manner to determine the contributions of the markers assessed by limited forward selection and backwards elimination.

Table 6-2. Marker combinations

Model	Tests included
A	APRI
Bi	APRI + ELF
Bii	APRI + TE
C	APRI + ELF + TE
D	APRI + ELF + TE + HTT

Abbreviations: APRI, aspartate transaminase to platelet ratio index; ELF, the enhanced liver fibrosis panel; TE, transient elastography; HTT, hepatic transit time.

6.5 Results

Initially, 80 subjects were recruited into the study. Antecubital vein cannulation was not possible in five subjects (all of whom had a history of intravenous drug use or self-harm), satisfactory liver stiffness measurement was not possible in five subjects (of whom two were obese and two were South Asian females with narrow intercostal spaces), three subjects had insufficient liver biopsy length (<10mm), leading to the exclusion of a total of 13 patients. The characteristics of the 67 patients included in the analysis are displayed in **Table 6-3**. The median length of time from liver biopsy to investigation was 4 months, and this did not vary significantly between groups (Kruskal-Wallis test, $p=0.41$).

Table 6-3. Patient characteristics

	Total	Mild fibrosis (stage 0-2)	Moderate fibrosis (stage 3-4)	Cirrhosis (stage 5-6)
Number	67	30	23	14
M/F	44/23	18/12	17/6	9/5
Age, years (range)	50 (19-65)	49.5 (19-63)	49.0 (27-65)	52.5 (38-65)
BMI, kg/m ²	23.8 (5.2)	23.2 (4.9)	25.2 (6.9)	26.1 (4.3)
Time, Bx to Ix, months	4 (5)	5 (6)	4 (4)	4.5 (4.25)
Length Bx, mm	24 (17)	25 (15.5)	23 (16.5)	25 (18.5)
ALT, iu/L [0-40]	87.5 (93.5)	52.5 (79.5)	87.0 (82.0)	107 (106)
AST, iu/L [0-40]	58.0 (57.0)	37.0 (31.3)	67.0 (47.0)	103 (81.5)
Platelets, x10 ⁹ /L [130-450]	214 (96.0)	242 (77.8)	206 (119)	177 (95.0)
Albumin, g/L [35-50]	42 (5)	42.5 (3)	41 (5)	39 (5.5)
Total Bilirubin, μmol/L [0-20]	11 (8)	11 (9.25)	11 (6)	15 (7)
Viral load, iu/L x10 ⁵	2.95 (12.5)	3.00 (12.1)	4.22 (13.1)	0.65 (11.9)

Values expressed as median (interquartile range) unless stated otherwise. Reference ranges of values are included in square brackets. Abbreviations: M, male; F, female; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; Bx, biopsy; Ix, investigation.

The output of each marker was compared to fibrosis stage (in three groups) (**Figure 6-1**). There was a monotonic increase of median APRI, ELF and liver stiffness measurement with increasing fibrosis stage, while there was a negative correlation between HTT and increasing fibrosis stage.

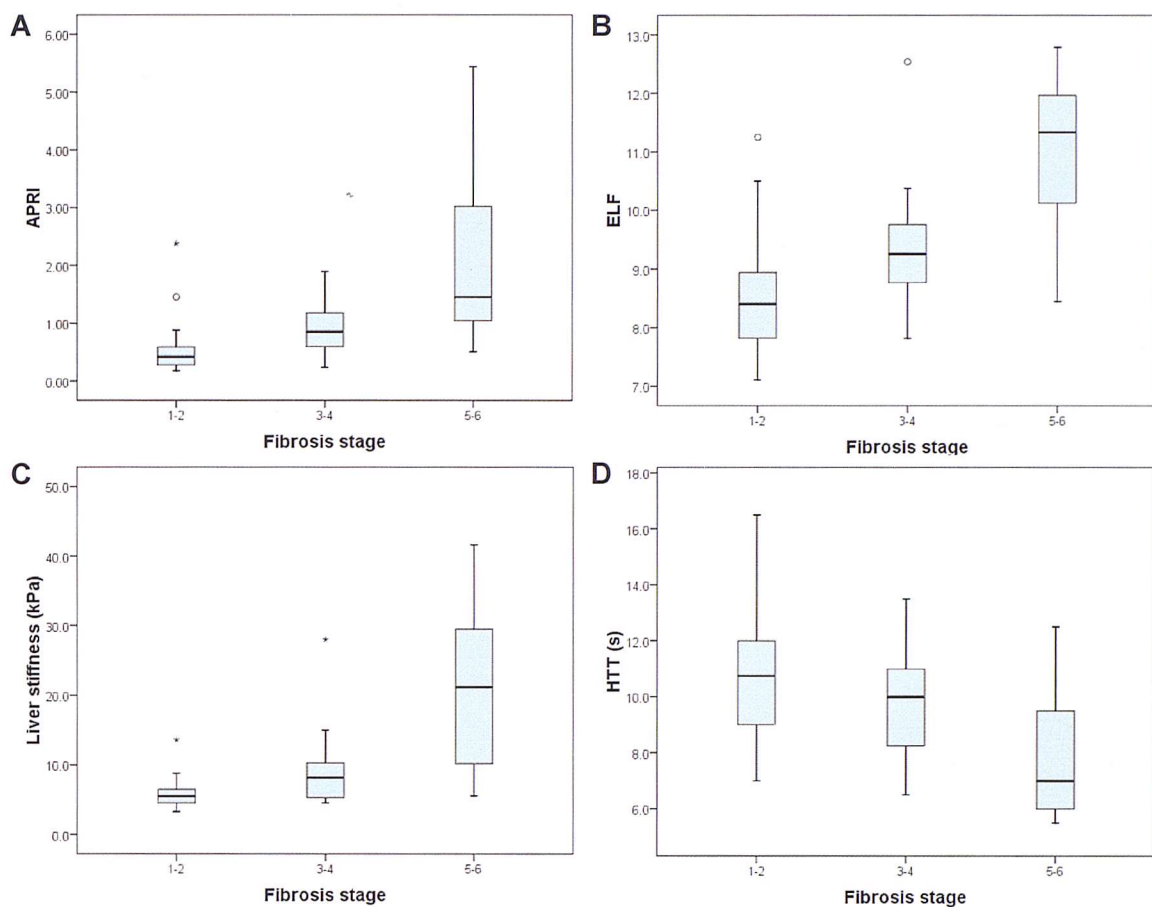


Figure 6-1. Marker scores plotted by fibrosis stage.

Categories are mild fibrosis (F1-2), moderate fibrosis (F3-4) and cirrhosis (F5-6). “Box and whisker” plots represent the median (horizontal line) and interquartile range (box) with the range of values up to 1.5 times the IQR within the range of each of the bars for the four markers with increasing fibrosis stage. Outliers (* & °) are plotted individually beyond the bars. **A:** APRI; **B:** ELF; **C:** TE; **D:** HTT. Abbreviations: APRI, aspartate aminotransferase to platelet ratio index; ELF, Enhanced Liver Fibrosis panel; TE, transient elastography; HTT, hepatic transit time.

ROC curves for each test for the diagnosis of cirrhosis and moderate to severe fibrosis demonstrate similar diagnostic performance for ELF and TE for the diagnosis of cirrhosis and of APRI, ELF and TE for the prediction of moderate and more severe fibrosis, compared to mild disease (**Figure 6-2**).

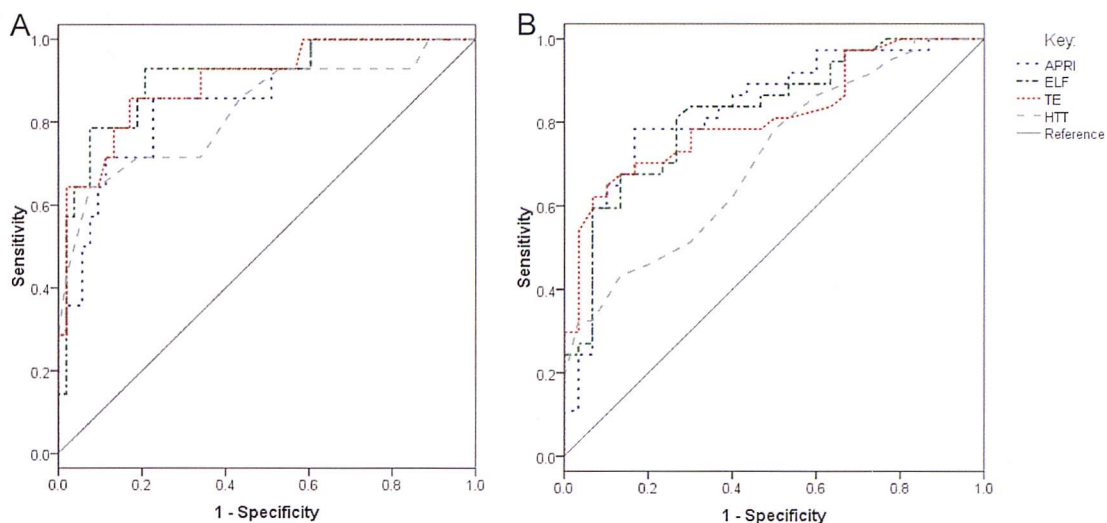


Figure 6-2. ROC curves show the diagnostic accuracy of each marker.

Panel *A* represents the diagnosis of cirrhosis (stage 5-6) and *B*, the diagnosis of fibrosis stage >2 (moderate to severe fibrosis). Corresponding AUCs and confidence intervals are displayed in **Table 6-4**. Abbreviations: APRI, aspartate aminotransferase to platelet ratio index; ELF, Enhanced Liver Fibrosis panel; TE, transient elastography; HTT, hepatic transit time. Diagonal lines represent ties.

The diagnostic accuracy, derived from the AUROC, with 95% confidence intervals, sensitivity, specificity, positive predictive value and negative predictive value for the markers in this cohort are displayed in **Table 6-4**. ELF and TE both demonstrated high diagnostic accuracy for the prediction of cirrhosis. When using the optimal cut-off for each test, ELF demonstrated higher sensitivity and TE, higher specificity. Using these cut-off points, the negative predictive value was high; from 92% for HTT to 98% for ELF. However, the relatively small number of patients with cirrhosis contributed to relatively low positive predictive values (ranging from 50-67%).

Table 6-4. Diagnostic accuracy of tests.

Total n=67	APRI	ELF	TE	HTT
Cirrhosis				
(stage >4), n=14				
AUC	0.86	0.91	0.90	0.83
95% CI	0.75-0.97	0.82-1.00	0.81-0.99	0.69-0.97
Cut-off	0.92	9.4	10.0	8.0
Se (%)	86	93	79	71
Sp (%)	77	79	87	91
PPV (%)	50	54	61	67
NPV (%)	95	98	94	92
Moderate-severe				
(stage >2), n=37				
AUC	0.83	0.82	0.82	0.71
95% CI	0.73-0.93	0.73-0.92	0.71-0.92	0.59-0.84
Cut-off	0.66	8.75	8.0	10.25
Se (%)	83	84	90	53
Sp (%)	78	70	65	73
PPV (%)	76	78	68	62
NPV (%)	85	78	89	66

Cirrhosis is defined as Ishak fibrosis stage 5-6 compared to stage <5, with positive state being cirrhosis.

Moderate to severe fibrosis is defined as Ishak fibrosis stage >2 compared to stage 1-2, where the positive state is stage >2. Cut-off values were derived from the ROC curves, where the sum of the sensitivity and specificity was maximal. Abbreviations: AUC, area under (the receiver operating characteristic) curve; APRI, aspartate aminotransferase to platelet ratio index; ELF, the enhanced liver fibrosis panel; TE, transient elastography; HTT, hepatic transit time; F, fibrosis stage; AUC, area under the curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

Reliability was assessed using the intraclass correlation coefficient on 26 patients, who were assessed on two occasions, separated (**Table 6-5**). This showed that ELF and TE were the most reliable tests with an ICC of 0.94 apiece, demonstrating that the variability in the dataset was much greater between patients than between visits. HTT was least reliable; ICC, 0.80.

Table 6-5. Reliability of markers

	ICC	95% CI
APRI	0.91	0.81-0.96
ELF	0.94	0.88-0.97
TE	0.94	0.87-0.97
HTT	0.80	0.61-0.91

Abbreviations: APRI, aspartate aminotransferase to platelet ratio index; ELF, the enhanced liver fibrosis panel; TE, transient elastography; HTT, hepatic transit time.

Each marker was applied to three-group models of disease severity; the goodness of fit, accuracy and significance are displayed in **Table 6-6**. This demonstrated that each test was a highly significant predictor of fibrosis and that while ELF demonstrated the highest goodness-of-fit, TE showed the highest diagnostic accuracy for the prediction of fibrosis. ELF was the strongest predictor of necro-inflammation, while TE did not significantly predict necro-inflammation. The accuracy of all marker combinations for assessment of necro-inflammation was substantially lower than those of fibrosis.

Table 6-6. Individual marker performance assessed using a three-group analysis

Marker	Goodness- of-fit	Accuracy %	Estimate	95% CI	Significance (p=)
<i>Fibrosis</i>					
APRI	0.84	64.2	2.45	1.30-3.60	<0.0005
ELF	0.77	65.7	1.33	0.81-1.85	<0.0005
TE	0.80	68.7	0.28	0.14-0.41	<0.0005
HTT	0.90	52.2	-0.50	-0.75- -0.25	<0.0005
<i>Inflammation</i>					
APRI	1.04	49.3	0.68	0.14-1.22	0.014
ELF	1.02	53.7	0.53	0.19-0.88	0.003
TE	1.04	35.8	0.05	-0.005-0.11	0.075
HTT	1.30	40.3	-0.24	-0.44- -0.036	0.021

Goodness of fit was calculated as the deviance divided by the number of degrees of freedom. Accuracy is the predicted class divided by the actual class, expressed as a percentage. Abbreviations: APRI, aspartate aminotransferase to platelet ratio index; ELF, the enhanced liver fibrosis panel; TE, transient elastography; HTT, hepatic transit time.

The results of the step-wise ordinal regression analysis are displayed in **Table 6-7**. Both ELF and TE contributed significantly to two-test combinations (Bi and Bii), which included the APRI score, with an accuracy of 70% for the prediction of fibrosis stage within a three-stage scale. A three-test combination (C) increased the goodness-of-fit and accuracy, although the individual tests were not independently significant contributors to the model. For the prediction of necro-inflammation, ELF was a significant contributor to combinations C and D, with the optimal accuracy 61% for model C.

Table 6-7. Performance of marker combinations assessed using a three-group analysis

Model	Goodness- of-fit	Accuracy (%)	APRI p=	ELF p=	TE p=	HTT p=
<i>Fibrosis</i>						
A	0.84	64.2	<0.0005			
Bi	0.75	70.1	0.045	0.002		
Bii	0.76	70.1	0.018		0.006	
C	0.74	73.1	0.079	0.095	0.203	
D	0.74	74.6	0.089	0.151	0.251	0.456
<i>Necro- inflammation</i>						
A	1.04	49.3	0.014			
Bi	1.02	53.7	0.509	0.058		
Bii	1.05	49.3	0.071		0.932	
C	1.00	61.2	0.196	0.010	0.081	
D	1.00	53.7	0.179	0.026	0.065	0.356

Marker performance tabulated according to goodness-of-fit and accuracy of marker combinations in predicting three-group models of fibrosis and necro-inflammation. Figures in bold type are statistically significant. Abbreviations: APRI, aspartate aminotransferase to platelet ratio index; ELF, the enhanced liver fibrosis panel; TE, transient elastography; HTT, hepatic transit time.

6.6 Discussion

6.6.1 Novel findings

This is the first study to compare the diagnostic performance of ELF and HTT with other markers of the severity of chronic liver disease and with each other. For the diagnosis of cirrhosis, ELF performed as well as TE, and better than APRI. ELF performed as well as both TE and APRI for the diagnosis of moderate to severe fibrosis. The diagnostic accuracy for HTT was substantially lower than that of the other three markers. In addition, HTT did not contribute significantly to the marker combinations for either fibrosis or inflammation, despite the modest increase in accuracy. HTT was also the least reliable test, with an ICC of 0.8 (compared to reliability in excess of 90% for the other markers).

This is also the first study to combine markers explicitly on the basis of cost and practicality. As increasing numbers of biomarkers have been shown to predict liver disease severity, such considerations will help to determine which markers enter clinical practice or are employed in clinical trials.

The novel three-group ordinal regression analysis enabled the interactions between markers to be investigated in combinations on the basis of increasing cost and complexity. A single cheap test, the APRI score, correctly predicted 92% of those correctly predicted by a two-test model including either ELF or TE and 88% of those correctly predicted by a three-test model including both TE and ELF.

Scrutiny of the multi-marker models allowed an assessment of the interplay between tests when predicting the histological stage. When a component of the combination was significant, its removal from the model significantly altered the goodness-of-fit of that particular model; those tests were considered complementary. If there was no significant change on removal of a test, it was considered redundant. For example, both ELF and TE significantly enhanced a two-test

model of fibrosis containing the APRI score, while neither ELF nor TE contributed significantly to the three-test model provided by the other two tests (**Table 6-7**). This does not imply that either test is not a predictor of fibrosis, but that its inclusion in that model did not provide additional information to the other tests; that is, there was a degree of redundancy.

As this was a head-to-head analysis without missing data, systematic bias between tests was minimised. The tight exclusion criteria and well-matched patient groups minimise potentially confounding factors. The analysis approach enables the contribution of each test to the accumulated combinations to be quantified.

6.6.2 Results in context of previous studies

The diagnostic accuracy achieved by the tests, derived from ROC curves for the diagnosis of cirrhosis from less severe fibrosis, was comparable to that found previous studies using TE and/or APRI. The diagnostic accuracy for the separation of mild from more severe fibrosis exceeded 80% for APRI score, ELF panel and TE, but only just exceeded 70% for HTT (Castera *et al.*, 2005; Parkes *et al.*, 2006a; Parkes *et al.*, 2006b; Shaheen and Myers, 2007). Demonstration of similarity in results, using conventional analysis of markers that have been studied previously, validates the cohort used in this study and suggests that the novel findings would be reproducible if extended to other cohorts. The use of conventional analysis also provides a context for the assessment of marker combinations using ordinal regression.

Previous publications have proposed algorithms where agreement or concordance between tests of similar diagnostic accuracy lent confidence to the interpretation of a binary result and proposed the avoidance of diagnostic liver biopsy. Where results were discordant, biopsy was proposed (Castera *et al.*, 2005). By contrast, this current study provides evidence on whether tests are complementary or redundant. This may enhance the building of optimal combinations of tests. Furthermore, if tests are complementary, agreement will reflect the disease state. If tests

are redundant, agreement may simply reflect that the tests are measuring a similar parameter, which may or may not be closely related to the disease state or reference standard.

The performance of HTT should be discussed in the context of previous studies using the technique. An earlier study using microbubble ultrasound on a cohort of patients with chronic hepatitis C demonstrated apparently excellent separation of histologically-classified disease severity groups (Lim *et al.*, 2005b). However, there were a number of differences between that study and the current study. Firstly, the previous studies compared groups stratified by a combination of fibrosis stage and necroinflammatory score. The inflammatory component increased the separation of groups. Therefore, this was not strictly a study to predict liver fibrosis and the results were not readily comparable to analyses of other markers, as both classification and statistical methods differed from those used by other groups. Secondly, the previous study used an older-generation microbubble contrast agent, Levovist, which had a slower transit time than Sonovue[®], used in the current study (Lim *et al.*, 2006). Mechanisms for the differences in transit times between agents have not been established, but microbubble size, homogeneity, durability and potential microbubble surfactant-to-endothelial interaction (“stickiness”) all differ between agents. These factors may influence the pharmacokinetics of the agent, leading to differences in microbubble tropism (Lim *et al.*, 2004). Thirdly, the newer ultrasound hardware and vascular recognition software have resulted in increased image sensitivity. Fourthly, the HTT calculation used in the current study (**section 6.4.2.4**) differs from the H/TT, used historically in the publications by Lim and colleagues, in which the time from microbubble injection to appearance in the hepatic vein was used. The current calculation, used at Imperial College London and elsewhere, has evolved with the changes listed above (Searle *et al.*, 2008).

6.6.3 Validity of the patient cohort

Biopsy criteria in many centres lead to the under-representation of patients with mild disease, where histology is the reference standard compared to the patient population as a whole.

Moreover, a significant minority of patients with normal transaminase tests, who are less likely to undergo biopsy, are found to have a substantial degree of fibrosis (Sebastiani *et al.*, 2008). Patients with clinically overt or decompensated cirrhosis of known aetiology do not usually require biopsy for clinical purposes, so the full spectrum of diseases is unlikely to have been fully represented. This selection bias may be present in the current study population, potentially affecting ROC curves and values of sensitivity, specificity, positive predictive value and negative predictive value (Poynard *et al.*, 2007). However, the ordinal regression model presented is robust to such effects, since strict cut-off values are not used and assumptions about disease state prevalence are not used. Moreover, as pointed out in **section 6.6.2**, the results for individual markers are similar to previous publications. This raises the possibility that other studies may be similarly biased.

6.6.4 The contribution of inflammation to markers of fibrosis

It has been shown previously that inflammation increases liver stiffness (**section 1.7.3.5**). Furthermore, APRI is likely to be affected by inflammation on account of the hepatic AST component. Indeed, the ordinal regression analysis for each individual marker demonstrates that ELF is the most significant predictor of inflammation, with APRI and HTT also proving to be significant predictors. The predictive ability of TE for the assessment of inflammation was not significant in this cohort, although a number of studies have shown that inflammation profoundly affects liver stiffness in cohorts of patients where inflammation is the predominant pathology (Arena *et al.*, 2008; Coco *et al.*, 2007; Sagir *et al.*, 2008). Although there was a significant correlation between fibrosis score and NI score in this cohort of patients (Spearman; $r=0.3$, $p=0.018$), these findings suggest that all markers of fibrosis are likely to be influenced by inflammation. Thus, the presence of co-existent inflammation must always be considered when predicting fibrosis stage from biomarker scores.

6.6.5 Practicality of markers

Practical differences between markers, including cost, staffing and technique availability are highlighted in **Table 6-1**. Where the diagnostic performance of markers is equivalent, such considerations may influence their future use clinically and in the research setting. APRI is clearly cheap, simple and would not require further samples in addition to those used for routine clinical purposes. TE is quick, simple, effective and provides real-time information, requiring a single operator. It is easily applied if the relatively expensive hardware is in place. ELF is easy to acquire, but expensive on a sample-by-sample basis. HTT involves ongoing costs for consumables (microbubbles) and requires the availability of high specification ultrasound hardware, specialist software and two operators. However, HTT may be performed at the time of abdominal ultrasound examination, which is a part of routine assessment of patients with chronic liver disease, thus reducing the additional costs. Examination of the dynamic enhancement of the hepatic parenchyma increases the detection of hepatocellular carcinoma (Leen *et al.*, 2006; Quaia *et al.*, 2004), which is a major complication in patients with cirrhosis, but is also seen in pre-cirrhotic disease, particularly in patients with chronic hepatitis B virus infection. Such a technique may, therefore, be an appropriate addition to APRI in those without access to TE or ELF markers, and in cohorts of patients where imaging of the liver is required, particularly where there is an increased risk of hepatic malignancy.

6.6.6 Limitations and solutions

The most prominent limitation of this study was the use of liver biopsy with inherent sampling variability (Regev *et al.*, 2002), itself a surrogate marker of disease, as a reference standard. Sampling variability was minimised by the exclusion of samples <10mm in length. Inter-observer variability was minimised by the review of all biopsy specimens by an experienced hepatic histopathologist. There was a delay from biopsy to investigation of 4 months in this cohort. However, there was no difference in delay between groups. In addition, studies have demonstrated that in chronic hepatitis C, it is unlikely for a significant change in fibrosis stage

to be observed within 3-5 years (Marcellin *et al.*, 2002; Poynard *et al.*, 1997; Wright *et al.*, 2003). The necroinflammatory score may indeed change over a number of months, but as there was no difference in delay between groups, such changes apply to all the tests evaluated. However, such short term inflammatory changes may result in an underestimation of marker performance for the prediction of inflammation in this study. In ideal circumstances, however, only biopsies in excess of 20mm, with 11 or more complete portal tracts would be used, and biopsies would be performed on the day of investigation. However, given current clinical circumstances, such a recruitment strategy would have resulted in smaller numbers of subjects included in the study. Analysis of histology might also be performed by two blinded histopathologists independently, but these resources were unavailable for the purposes of this study.

This study was limited by a relatively small number of patients included in the analysis. Patients with decompensated cirrhosis were excluded from the study and patients with established cirrhosis of known aetiology may not require liver biopsy, as the diagnosis may be made clinically. Accordingly, the pool of patients with cirrhosis who were eligible for inclusion in the study may not represent the full spectrum of disease. Of those with mild disease, there may be patients without deranged liver function tests in whom hepatitis C is either not diagnosed or in whom a liver biopsy is not considered a clinical priority. Therefore, care should be taken when extrapolating the results of this study to the wider population. In addition, for these reasons, the creation of clinical algorithms was not considered appropriate.

6.6.7 Future studies

This study has examined marker performance in a cohort of patients with chronic hepatitis C. For these results to be generalisable to chronic liver disease as a whole, their performance should be assessed for the prediction of disease severity in other disease cohorts, including chronic HBV infection, alcoholic liver disease and NAFLD. While this study provides novel information about the relative diagnostic performance of four markers, singly and in

combination, a formal cost-benefit analysis would allow further appraisal of these tests for widespread incorporation into clinical usage. Cost-benefit analysis would require estimation of clinical events saved by the use of non-invasive tests and should incorporate economies of scale. Such data were unavailable for this study. Consideration should also be made of potential access to hardware, appropriately-trained personnel or external laboratory services. Attempts to predict clinically-important endpoints prospectively using marker combinations are warranted. A longitudinal study assessing the change in these markers in response to antiviral treatment with pegylated interferon and ribavirin in a cohort of patients with chronic hepatitis C is underway and results are awaited.

6.7 Conclusions

To conclude, this impartial head-to-head analysis of the diagnostic performance of two serum and two imaging-based markers of fibrosis shows that ELF and TE have a high diagnostic accuracy for the prediction of fibrosis, and that HTT performs less well than the other tests. A combination of APRI with either ELF or TE effectively predicts fibrosis stage using a three-group model of fibrosis, but that, in combinations of three or more tests leads to redundancy of information. This study may provide a basis for future clinically-based cost-benefit analyses to assess optimal combinations of markers.

Finally, the work presented in this chapter has defined analysis techniques, which will enable the effectiveness of candidate markers (such as those conveyed by ^1H MRS) to be assessed by comparison with the most effective biomarkers of liver disease severity in CHC in the next chapter.

7 ¹H MRS IN THE CONTEXT OF OTHER NON-INVASIVE TECHNIQUES

7.1 Abstract

In preceding chapters, ¹H MRS for the assessment of chronic liver disease has been developed *in vitro* and applied to patients with CHC *in vivo*. MR indices of lipid composition and choline have been shown to correlate with disease severity. In Chapter 6, two imaging-based markers and two serum markers were assessed head-to-head and in combination to determine their relative performance and the interactions between them. The purpose of this chapter was to evaluate the performance of MR indices derived and validated in preceding chapters for the assessment of disease severity in CHC, compared to established biomarkers validated in this thesis and elsewhere.

38 patients with biopsy-proven chronic hepatitis C were recruited into the study. All patients had ¹H MRS, TE and APRI. The MR indices used were the polyunsaturation index (PUI) and the total choline to lipid ratio (CLR). In binary analyses, PUI and CLR predicted cirrhosis with a diagnostic accuracy of 83% and 76% and mild from more severe disease with a diagnostic accuracy of 77% and 79% respectively. The accuracy for the prediction of cirrhosis was less than that of TE but similar to that of APRI. For the separation of mild from more severe disease, the accuracy was less than that of either APRI or TE. Using ordinal regression, either PUI or CLR significantly contributed to the prediction of fibrosis stage when combined with APRI. When combined with both APRI and TE, PUI or CLR significantly contributed to the model, where TE did not make a significant contribution.

Conclusion: the MR indices predicted fibrosis with less accuracy in this study than leading markers currently available. Combination of these indices with APRI improved the model, but combination with APRI and TE led to redundancy of information from TE.

7.2 Introduction

In preceding chapters, ^1H MRS for the assessment of chronic liver disease has been developed *in vitro* and applied to patients with chronic hepatitis C *in vivo*. MR indices have been derived on the basis of pattern-recognition analysis of MR spectra of intact liver tissue and from the literature (**sections 4.4.2.1 and 4.5.1**). These indices have been shown to correlate with disease severity in patients with chronic hepatitis C. In **Chapter 6**, two imaging-based markers (HTT and TE) and two serum markers (APRI and ELF) were assessed head-to-head, and in combination, to determine their relative performance and the interactions between them. Individually, TE or ELF performed best for the prediction of histological fibrosis stage, while combination with APRI improved the performance of each. The aim of this chapter was to evaluate the performance of these MR indices for the assessment of disease severity in chronic hepatitis C, compared to APRI and TE.

Two ^1H MRS indices were selected. The PUI is an MR-detectable measure of fatty acid polyunsaturation within the region of interest. As discussed in **section 4.5.1**, reduced polyunsaturation is associated with increasing severity of fibrosis and increased hepatic steatosis. Polyunsaturated fatty acids are protective against oxidative stress and are consumed by lipid peroxidation. Tissue environments subject to oxidative stress are associated with cellular damage, inflammation and scarring or fibrosis (**section 1.5.3**). As such, PUI might be considered a surrogate marker of disease severity, providing an insight into pathogenically-relevant processes not assessed by current non-invasive markers. The choline to lipid ratio (CLR) was established from the regression vector of the partial least squares discriminant analysis (PLS-DA) of *in vitro* MRS resonances, which were most associated with increased severity of fibrosis (**section 4.4.2.1, Table 4-2**). Biologically, the total choline resonance consists of the functional group comprising a number of choline-related compounds, including choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, TMAO and betaine. The resolution of ^1H MRS *in vivo* is insufficient to observe each constituent, but the choline region itself is likely to be related to membrane metabolism and cell-turnover (Katz-Brull *et al.*, 2002;

Ruiz-Cabello and Cohen, 1992; Taylor-Robinson *et al.*, 1995). Like liver stiffness measurement, such a marker is a blunt correlate of disease, of which the contributing factors are not clearly delineated. Although PUI has been observed in the context of hepatic steatosis, neither PUI nor CLR has previously been applied to chronic fibrotic liver disease, or compared to other markers of disease severity.

7.3 Materials and methods

7.3.1 Patients

Ethical approval for the study was obtained from the regional ethics committee and the study was conducted in accordance with the Declaration of Helsinki. A total of 85 patients with chronic hepatitis C were recruited prospectively from the Liver Unit at Imperial College Healthcare Trust from March 2006 to January 2008. The patient cohorts for the analyses described in **Chapters 5-7** were all taken from this total. All gave written, informed consent to participation in the study. The inclusion and exclusion criteria are specified in **sections 5.5.1 and 6.4**. ^1H MRS was performed on a subset of these patients ($n=59$). In order to maximise the number of patients eligible for inclusion in the study and to include a range of modalities, APRI and TE were chosen for comparison to the MR indices. Of these patients, 38 had satisfactory PUI, CLR, APRI and TE measurements, so were included in this analysis.

7.3.2 Histology

Percutaneous liver biopsy was obtained from all participants in the clinical setting and all samples were reassessed by a single, blinded hepatic histopathologist according to the modified histological activity index (Ishak *et al.*, 1995), as described in **section 1.6.3.1**. Patients were excluded if their biopsy was shorter than 10mm. All patients underwent venesection on the day of ^1H MRS examination.

7.3.3 Non-invasive markers

All blood samples and imaging tests were obtained on the same visit and within 2hr of each other. Patients fasted overnight or for a minimum of 6hr prior to the investigations.

7.3.3.1 *APRI*

The APRI was defined as described previously and derived from routine clinical blood tests (**section 6.4.2.1**).

7.3.3.2 *Liver stiffness measurement*

Liver stiffness measurement was obtained by TE as previously described (Ziol *et al.*, 2005). Criteria for satisfactory measurements are described in **section 6.4.2.3**.

7.3.4 Statistics

SPSS v16 (SPSS Inc. Chicago, USA.) was used for statistical modelling. The statistical techniques used were described in **section 6.4.3**. As both the PUI and CLR included the total MR-visible lipid resonances as the denominator, they were not analysed in combination. All other combinations of the markers tested were considered. Correction for multiple comparisons was not performed as the markers used in this chapter have been shown to be correlated to each other in **Chapters 5 and 6**.

7.4 Results

The patient characteristics of the cohort are displayed in **Table 7-1**. The distribution of patients according to fibrosis stage is skewed towards mild disease. The median biopsy length was 26mm, ranging from 12mm to 49mm, representing adequate sample size. The ALT and AST increase with severity of disease, and platelets and cholesterol are lower in the cirrhosis than the mild category, which is consistent with clinical experience.

Table 7-1. Patient characteristics

	Total	Mild fibrosis (stages 1-2)	Moderate fibrosis (stages 3-4)	Cirrhosis (stages 5-6)
Number	38	19	13	6
Sex M/F	21/17	11/8	8/5	2/4
Age, years (range)	49 (19-65)	50 (19-63)	48 (27-60)	52 (39-65)
Bx length, mm (range)	26.0 (12.0-49.0)	29.0 (13.0-48.0)	21.5 (12.0-49.0)	28.0 (15.0-38.0)
BMI, kg/m ²	23.0 (4.7)	22.5 (4.44)	24.9 (8.22)	25.1 (4.49)
ALT, iu/L [0-40]	73.0 (106.0)	50.0 (58.0)	101.0 (92.5)	160.0 (197.5)
AST, iu/L [0-40]	58.5 (60.5)	36.0 (29.0)	80.0 (48.5)	114.5 (143.0)
Platelets, x10 ⁹ /L [130-450]	208.0 (98.0)	210.0 (76.0)	170.0 (117.5)	172.0 (93.5)
tChol, mmol/L	4.33 (1.26)	4.50 (1.08)	4.68 (1.56)	3.55 (1.21)
TG, mmol/L	0.95 (0.61)	0.91 (0.64)	0.96 (0.63)	1.00 (0.20)

Values expressed as median (interquartile range) unless stated otherwise. Reference ranges of values are included in square brackets. Abbreviations: M, male; F, female; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; Bx, biopsy; Ix, investigation; tChol, total cholesterol; TGs, triglycerides

The indices were plotted by severity of fibrosis in **Figure 7-1**, demonstrating that the trends of APRI and TE to increase with disease severity in this subset were similar to that of the larger, overlapping cohort in **Chapter 6 (Figure 6-1)**. PUI and CLR both decreased with increasing disease severity, as described for the “parent” cohort in **Chapter 5 (Figure 5-3)**.

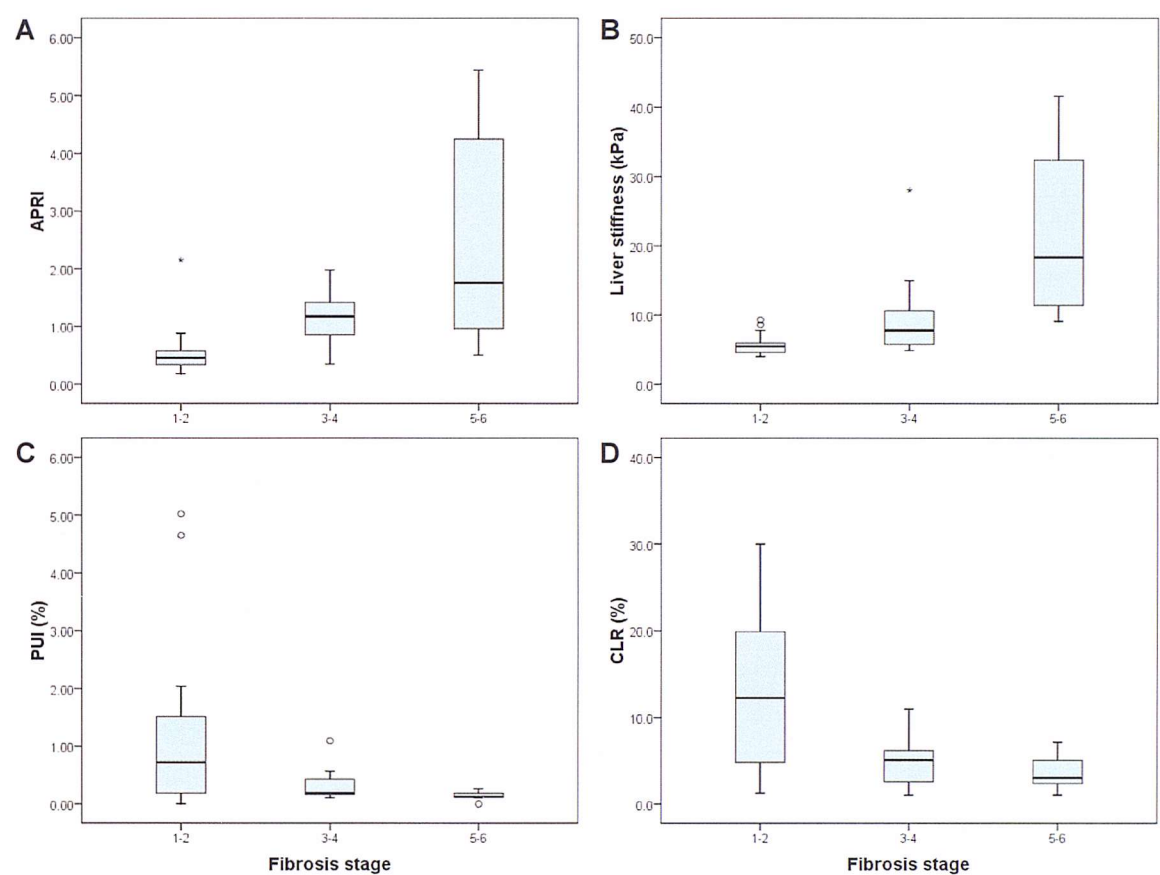


Figure 7-1. Marker scores plotted against fibrosis stage.

“Box and whisker” plots represent the median (horizontal line) and interquartile range (box) with the range of values up to 1.5 times the IQR within the range of each of the bars, for the four markers with increasing fibrosis stage. Outliers are plotted individually beyond the bars. A: APRI; B: TE; C: PUI; D: CLR. Abbreviations: APRI, aspartate aminotransferase to platelet ratio index; TE, transient elastography; PUI, polyunsaturation index; CLR, total choline to lipid ratio.

The ROC curves in **Figure 7-2** show graphically that, for the diagnosis of cirrhosis, TE outperformed the other markers, which performed similarly to each other in this cohort. For the diagnosis of moderate to severe disease, both APRI and TE had a higher AUROC than the MR

indices. Using cut-off values derived from the ROC curves, optimal sensitivity, specificity, PPV and NPV are displayed for this cohort in **Table 7-2**. As the number of patients in the cirrhosis group was small in this cohort, the PPV for cirrhosis was low for all tests.

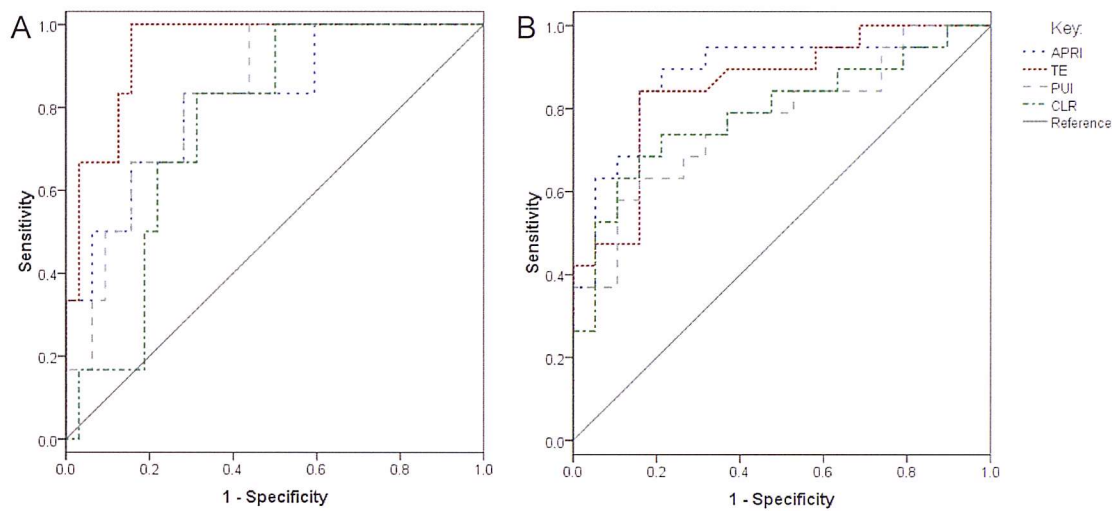


Figure 7-2. ROC curves show the diagnostic accuracy of each marker.

Panel **A** shows ROC curves for the diagnosis of cirrhosis (stage 5-6) and **B**) the diagnosis of fibrosis stage >2 (moderate to severe fibrosis). Corresponding AUCs and confidence intervals are displayed in **Table 7-2**. Abbreviations: APRI, aspartate aminotransferase to platelet ratio index; TE, transient elastography; PUI, polyunsaturation index; CLR, total choline to lipid ratio. Diagonal lines represent ties.

Table 7-2. Diagnostic accuracy of tests

Total n=38	APRI	TE	PUI	CLR
Cirrhosis (stage >4), n=6				
AUC	0.82	0.94	0.83	0.76
95% CI	0.63-1.00	0.87-1.00	0.68-0.98	0.60-0.92
Cut-off	0.92	9.0	0.19	5.1
Se (%)	83	100	83	83
Sp (%)	72	84	72	69
PPV (%)	36	55	36	33
NPV (%)	96	100	96	96
Moderate-severe (stage >2), n=19				
AUC	0.88	0.86	0.77	0.79
95% CI	0.77-1.00	0.73-0.98	0.62-0.92	0.64-0.94
Cut-off	0.65	6.2	0.24	7.0
Se (%)	84	84	74	74
Sp (%)	84	84	68	68
PPV (%)	84	84	70	70
NPV (%)	84	84	72	72

Abbreviations: APRI, aspartate aminotransferase to platelet ratio; TE, transient elastography; PUI, polyunsaturation index; CLR, total choline to lipid ratio; AUC, area under the curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

The ordinal regression showed the correct prediction of one of three fibrosis severity categories, using the markers singly and in combination. When looking at all three categories, the APRI performed best in terms of the lowest figure for goodness-of-fit, and the highest number of patients with the fibrosis category correctly predicted (**Table 7-3**). These figures are comparable to those displayed in **Table 6-6**, demonstrating that this cohort is representative of the larger patient cohort in **Chapter 6**.

In combination, it can be seen that, although the goodness-of-fit improved with the addition of successive markers, the diagnostic accuracy was optimal with a combination of APRI and TE or

PUI. PUI and CLR contributed significantly to the marker models, suggesting that in these cases, the markers were complementary. The addition of TE to combinations which included APRI and one of the MR indices did not significantly enhance the model, so this information may be considered redundant.

Table 7-3. Ordinal regression of individual markers for the prediction of fibrosis

Marker	Goodness of fit	Accuracy %	Regression Estimate	95% CI	Significance (p=)
APRI	0.79	71.1	2.00	0.77-3.23	0.002
TE	0.85	63.2	0.24	0.08-0.39	0.003
PUI	0.87	55.3	-2.18	-4.00- -0.36	0.019
CLR	0.87	63.2	-0.19	-0.31- -0.06	0.004

The model is based on three fibrosis categories: mild (stages 0-2), moderate (stages 3-4) and cirrhosis (5-6). Abbreviations: CI, confidence interval; APRI, apsartate aminotransferase to platelet ratio index; TE, transient elastography; PUI, polyunsaturation index; CLR, total choline to lipid ratio

Table 7-4. Diagnostic performance of combinations of markers

Model	Goodness-of-fit	Accuracy (%)	APRI p values	TE p values	PUI p values	CLR p values
APRI	0.79	71.1	0.002			
APRI+TE	0.72	73.7	0.022	0.040		
APRI+PUI	0.68	73.7	0.003		0.043	
APRI+CLR	0.68	71.1	0.005			0.011
TE+PUI	0.70	68.4		0.010	0.094	
TE+CLR	0.69	71.1		0.008		0.015
APRI+TE+PUI	0.59	73.7	0.012	0.052	0.023	
APRI+TE+CLR	0.64	73.7	0.045	0.071		0.018

Ordinal regression of combinations of markers for the prediction of fibrosis severity. Fibrosis categories are mild (stages 0-2), moderate (stages 3-4) and cirrhosis (5-6). Abbreviations: CI, confidence interval; APRI, aspartate aminotransferase to platelet ratio index; TE, transient elastography; PUI, polyunsaturation index; CLR, total choline to lipid ratio.

7.5 Discussion

When considered as surrogate markers of fibrosis, both PUI and CLR have been shown to predict fibrosis category with moderate accuracy in this cohort. Moreover, in combination with a simple blood test, APRI, they enhanced the diagnostic accuracy further. Individually, however, they performed less well than either APRI or TE. However, it must be stressed that fibrosis is a pathologically-defined lesion, indicative of chronic liver injury and prognostically-related to risk of end-stage liver disease. Fibrosis, despite being undeniably relevant to chronic liver disease cannot be considered to define chronic liver disease entirely, as it is in itself a surrogate marker of liver disease. Aetiology, host-factors and other pathological lesions are all relevant to the natural history of liver disease and to the probability of response to treatment. Alternative biologically-relevant indices are required to provide a global assessment. The non-invasive assessment of hepatic lipid composition is one such aspect. Of the ^1H MRS indices assessed in this chapter, the PUI was selected as lipid polyunsaturation was hypothesised to be an important biological factor in chronic liver disease. CLR was selected as, on multivariate pattern recognition using PLS-DA, the choline and lipid resonances were found to be prominent predictors of disease severity in liver biopsy samples (**Chapter 4**).

The utility of new technologies in clinical practice depends not only on diagnostic performance, but also on cost effectiveness and practicality. These issues have been addressed for the ultrasound and serum markers in **Table 6-1** and **section 6.6.5**. For comparison, ^1H MRS is more expensive and time-consuming than either of APRI or TE; MR scans for academic research at the Robert Steiner MR Unit, Hammersmith Hospital Campus, London, UK were charged at £450 per patient study (2005-2008). On the evidence from this cohort, neither PUI nor CLR can be recommended as markers of fibrosis for routine clinical purposes. CLR in particular, is strongly influenced by total hepatic lipid, so would be confounded by major alterations in lipid levels. However, when considering markers of disease more broadly, it can be argued that steatosis is integral to the aetiology and progression of many chronic liver diseases (**Chapter 1**). Moreover, it has been discussed in **Chapters 3** and **4** how lipid composition and, in particular,

measures of lipid polyunsaturation are relevant to both NAFLD and chronic hepatitis C. That lipid composition differs with disease severity is an argument for its importance in chronic liver disease, rather than a suggestion that such an index is a direct marker of hepatic fibrosis. **Table 7-4** has demonstrated that PUI and CLR are complementary to APRI and TE for the prediction of fibrosis stage. More broadly, the non-invasive assessment of lipid composition may complement the assessment of other aspects of liver disease, in combination with other non-invasive techniques.

7.5.1 Limitations and solutions

7.5.1.1 *General limitations*

This study is limited by relatively small numbers of patients included in the analysis. In particular, there were only six patients in the cirrhosis group. Accordingly, as discussed in **section 6.6.6**, it is not appropriate to make firm conclusions about the relative performance of markers for the prediction of cirrhosis from this small number of patients. It is assumed that these patients are representative of patients with cirrhosis, which may not be the case. Patients with decompensated cirrhosis have been excluded from the study and patients with established cirrhosis of known aetiology may not require liver biopsy, as the diagnosis may be made clinically. Accordingly, the pool of patients with cirrhosis who were eligible for inclusion in the study does not represent the full spectrum of disease. The mild group contained 19 patients, making a comparison of techniques for the discrimination of mild from more severe disease more valid. Nevertheless, within the mild group, there may be those without deranged liver function tests in whom hepatitis C is either not diagnosed or in whom a liver biopsy is not considered a clinical priority. Therefore, care should be taken when extrapolating the results of such a small study to the wider population.

7.5.1.2 *Specific limitations*

Limitations specific to the individual techniques and the statistical methods used are discussed in the relevant sections. With respect to this study, it should be reiterated that the patients

included in the analysis overlap those studied for previous analyses. In addition, the PUI *in vivo* was calculated in 77% of the patients in **Chapter 5**, as the diallytic resonance was small and could not always be fitted. In this chapter, only those patients in whom all measurements were available were included. Therefore, while this serves as a useful comparison between markers, it does not constitute an independent validation of the techniques used.

7.5.2 Potential solutions

This study would be improved by a number of means. If a greater proportion of patients had had satisfactory measurement of all markers, there would be greater confidence in the comparison between tests. Optimal combinations could be suggested on the basis of such an analysis and should be validated using a separate cohort. The number of patients that have been recruited prohibits such an analysis. More even numbers of patients for each fibrosis stage would enable improved goodness-of-fit for the ordinal regression model of fibrosis stage.

7.6 Conclusions

This analysis suggests that the MR indices PUI and CLR are able to predict fibrosis category with accuracy comparable to other non-invasive markers. In combination, these MR indices complement APRI and TE. On the basis of the current study, neither PUI nor CLR can be recommended as a surrogate marker of hepatic fibrosis in the context of other currently available markers. However, the assessment of lipid composition and, specifically, PUI may be a biologically and pathologically-relevant adjunct to other techniques for the non-invasive assessment of chronic liver disease.

8 FUTURE DIRECTIONS

This thesis has addressed the feasibility and relevance of ^1H MRS for the assessment of chronic liver diseases and provides the basis for much future work. Further work may be divided into further technical development to improve the techniques used and applications of the techniques to validate them biologically and clinically.

8.1 Technical development

8.1.1 ^1H MAS NMR spectroscopy

The limitations of ^1H MAS NMR spectroscopy for the analysis of intact tissue include sampling variability and the effects of sample degradation. The sensitivity (defined by signal-to-noise) and resolution (defined by linewidth) could be improved by using stronger magnetic field strengths (up to 18T). As described in **section 2.2**, increased numbers of spectral acquisitions would also improve sensitivity, but to the potential detriment of spectral resolution and sample degradation. Spectral noise could be reduced by cooling the probe electronics, requiring upgraded hardware. Vibration control and atmospheric conditions should be tightly controlled by housing the magnet in a purpose-built room with an airlock, climate-control and an inflatable airbed. To ensure that the signal obtained is optimally quantitative, the sample should be confined to the centre of the magnetic field, better achieved using spherical sealing cell cavities.

8.1.2 Multivariate pattern recognition analysis techniques

The current approach to analysis of complex spectra with overlapping peaks is to “bucket” regions of the spectrum, prior to integration of the area under the curve within the bucket. Co-resonance of metabolites may lead to resonances being assigned inappropriately. Fitting of individual curves using jMRUI is not practical when a large number of resonances is seen. Targeted profiling is a new approach which fits the expected lineshape of metabolites to the

spectrum of interest, thereby enabling low-concentration metabolites to be distinguished from larger overlapping resonances (Weljie *et al.*, 2006). An application of this might be the subtraction of known lipid resonances from a spectrum, leaving the smaller metabolites unmasked. Sample-specific databases could then be used to fit resonances appropriate and relevant to the sample of interest, as the expected ratios of metabolites in tissue differ considerably from those seen in urine.

8.1.3 ^1H MRS *in vivo*

The major limiting factor in the application of *in vivo* ^1H MRS to chronic liver disease is inadequate resolution and SNR, which, in this context, has been shown to lead to the underestimation, or occasional failure to curve-fit, smaller resonances (**section 5.7.1.3**). Technical developments should, therefore, focus on increasing SNR and resolution. In theory, the SNR should increase proportionally to the increase in field strength (Edelstein *et al.*, 1986). ^1H MRS could be performed at 3T or higher and the spectral information obtained should be compared directly to that obtained at 1.5T. Additional factors include control of patient motion artefact, distorting magnetic susceptibility effects and hardware constraints, such as electronic “noise”. The demonstration that ^1H MRS is a biologically and clinically relevant tool may encourage manufacturers to invest in scanner development, with attention to these areas.

8.2 Biological

8.2.1 Lipid polyunsaturation and oxidative stress

Polyunsaturated fatty acids are consumed in conditions of oxidative stress to produce lipid peroxidation adducts. Oxidative stress is central to mechanisms of hepatic injury, steatosis and fibrosis, as discussed in **section 1.5.3**. In this thesis, evidence has been provided that indices of lipid polyunsaturation may be detected both *in vitro* and, non-invasively, *in vivo*. Moreover, polyunsaturation decreases with increasing fibrosis and steatosis, providing indirect evidence of a role in chronic liver disease. For PUI to be considered a potential non-invasive marker of oxidative stress in chronic liver disease, further work is required to relate directly PUFA levels with lipid peroxidation. To address this issue, a number of experiments could be performed. Firstly, semi-quantitative immunohistochemical staining for HNE and MDA adducts in liver tissue from biopsy adjacent to that used for ^1H MAS NMR spectroscopy could be correlated with the PUI, in collaboration with a histopathologist. However, such a study would be limited by the sampling variability inherent in any histologically-based study, by the semi-quantitative nature of immunohistochemistry and by the volatility of lipid peroxidation products, which may lead to poor sensitivity in biopsy samples, which have not been processed immediately upon acquisition.

Colorimetric assays for the quantitative assessment of lipid peroxidation products are available (Oxford Biomedical Research Inc. Oxford, MI, USA), although such assays are destructive, making the use of limited quantities of human tissue in such circumstances inappropriate. Furthermore, such assays would not be possible following ^1H MAS NMR spectroscopy analysis on account of the sample degradation. Such techniques could be employed, however, in the assessment of tissue from animal models of liver disease that are particularly associated with oxidative stress, such as carbon tetrachloride-induced liver injury. These experiments could also enable a dose-response curve of liver injury to lipid polyunsaturation to be established.

Controlled studies would enable changes associated with commencement and resolution of injury to be detected.

8.2.2 Lipid composition of hepatocyte models of HCV replication

Much work on viral mechanisms of replication and hepatic steatosis is performed using cell lines (Kapadia and Chisari, 2005; Miyanari *et al.*, 2007). Quantification of lipid is performed by electron or light microscopy, or by using chromatography techniques on cell extracts. ^1H MAS NMR spectroscopy of the intact cell fraction in cell-culture experiments would provide a rapid quantitative technique for the assessment of lipid composition, in which viral and culture-medium factors may be altered in a controlled fashion. Presentation of work contained in this thesis has resulted in an ongoing collaboration with virologists at Imperial College London to assess intracellular lipid in infected immortalised hepatocyte cell-lines infected with modified HCV, using ^1H MAS NMR spectroscopy.

8.2.3 Profiling of liver tissue subject to toxic, genetic and metabolic insults

In this thesis, metabolic profiling of intact liver tissue has been applied in murine models of NAFLD and in human liver biopsy samples in chronic hepatitis C. The development of robust and reliable protocols for data acquisition has formed the basis of new collaborations with GSK and the Biological Imaging Centre at Imperial College London for the investigation of the effects of α -naphthylisothiocyanate (ANIT), a toxin that causes biliary epithelial damage and cholestasis, on rat liver. ^1H MAS NMR spectroscopy of tissue forms part of a multi-modality, multi-system approach to the investigation of models of liver toxicity and biliary hyperplasia.

Another collaboration arising from this thesis is the study of tissue from murine models of obstetric cholestasis. The effect of pregnancy hormones on bile acid homeostasis is being investigated in murine models deficient in the nuclear receptor, farnesoid X (FXR), compared to controls in pregnant and non-pregnant mice fed on diets with or without cholic acid

supplementation (Van Mil *et al.*, 2007). Preliminary results have demonstrated marked differences in the lipid, choline and glucose regions between groups.

Further animal models of NAFLD are being developed using combinations of chemical mutagenesis and dietary manipulation. ^1H MAS NMR spectroscopy of liver tissue provides a rapid assessment tool that may aid high through-put phenotyping of the resultant models, in conjunction with biochemical and histological measures, and the proteomic and metabonomic profiling of biofluids.

8.2.4 Interventional studies

This thesis has demonstrated that the non-invasive assessment of lipid composition is feasible, in addition to measures of total hepatic lipid. As part of a study based at the University of Newcastle and in collaboration with investigators at Imperial College London, the effect of modulation of lipids with lipid-lowering agents and PUFA-rich fish oils is being studied in patients with chronic hepatitis C, who have failed to eradicate the virus with conventional antiviral therapy. A subset of these patients is undergoing hepatic ^1H MRS before and after lipid modulation. The effect of these interventions on lipid composition will be investigated longitudinally. The demonstration of changes in hepatic lipid in response to these interventions will further validate the technique. Furthermore, differences in hepatic and plasma lipids may be correlated with viral load and indicators of liver function.

8.3 Clinical applications

Hepatic lipid quantification using ^1H MRS has already been applied to thousands of patients in studies of the prevalence of hepatic steatosis in the USA (Browning *et al.*, 2004; Szczepaniak *et al.*, 2005). However, the relationship between simple hepatic steatosis and the development of progressive liver disease is still unclear. Thus widespread hepatic lipid quantification in the general population is not currently indicated.

It has been argued that the measurement of hepatic lipid composition is of importance in the pathogenesis of chronic liver disease. However, this needs to be demonstrated in prospective clinical studies.

Of the markers of hepatic fibrosis studied in this thesis, some, such as APRI and TE, are now widely available, have been extensively studied over the last 5 years, and are entering clinical practice. Their precise role will evolve with experience.

8.3.1 Validation of MR indices in CLD of differing aetiologies

The non-invasive assessment of hepatic lipid composition using ^1H MRS has been assessed in this thesis in a cohort of patients with hepatitis C-related chronic liver disease. Other investigators have recently assessed lipid composition in lean and obese subjects, although evidence of liver injury, in terms of inflammation or fibrosis, was not assessed and, as has been explained, their assignment of metabolites was incorrect (**section 5.2.1**).

The technique should be applied to patients groups in whom hepatic steatosis is thought to be a prominent factor in the development of disease. Thus, cohorts of patients with NAFLD and alcoholic liver disease should also be studied.

8.3.2 Combination of ^1H MRS with other MR sequences

MR techniques are being developed for the staging of chronic liver disease and show promise (Talwalkar *et al.*, 2008). As discussed in **section 1.7.3**, MRE to measure liver stiffness has demonstrated high accuracy for the assessment of fibrosis compared to biopsy, while producing a map of the spatial distribution of high stiffness regions (Huwart *et al.*, 2008). DWI stages fibrosis by assessing proton diffusion, thought to be reduced in fibrotic disease (Lewin *et al.*, 2007), while ^{31}P MRS has been shown to grade the severity of cirrhotic and precirrhotic liver disease by assessing phosphorus-containing membrane metabolites and energy intermediates (Lim *et al.*, 2003b). ^1H MRS sequences are quick and available on most clinical MR systems. Therefore, a multi-faceted assessment of chronic liver disease might be achieved by combining the assessment of lipid composition using ^1H MRS with MR sequences that provide information on cell membrane components, liver stiffness or measures of diffusion.

8.3.3 Development of therapeutic strategies for chronic liver disease

The elucidation of mechanisms of fibrogenesis has demonstrated a number of potential targets for drugs to slow, halt or reverse fibrosis and, potentially, cirrhosis. The lack of validated markers is a barrier to clinical studies of the effectiveness of novel therapies. In slowly progressive disorders, such as chronic liver disease, it may take many years for patients to develop the complications of cirrhosis, which are considered “hard” endpoints for clinical studies.

The pharmaceutical industry is under increasing pressure to reduce the costs of drug development as, currently, many drugs are developed but fail to progress through clinical trials. There is need to develop markers of “early signs of efficacy”. Such markers should be sensitive to change, but do not necessarily need to be mechanism-specific. The purpose of such markers is to enable drugs that do not show signs of efficacy to be excluded at an early stage in development, thus saving costs. Candidate drugs passing this hurdle can then be assessed in

longer term clinical studies using validated endpoints (Drs W. Vennart & B. Souberbelle, Pfizer UK Ltd. personal communication).

Longer term studies are also required that compare markers with hard endpoints, such as repeated histological assessment (with appreciation that histological assessment of liver biopsy is also a surrogate marker of liver disease) and the development of complications of cirrhosis, such as portal hypertension, the onset of hepatic encephalopathy and the development of hepatocellular carcinoma.

8.3.4 Markers of response to antiviral therapy

As part of the drive to develop early markers of efficacy, a subset of the patients studied in this thesis is currently being assessed longitudinally, over a period of 2 years, in response to antiviral therapy with pegylated interferon and ribavirin. The study is due for completion in October 2009. Having assessed marker performance cross-sectionally in **Chapters 6 & 7**, markers will also be compared in their ability to detect change associated with a response to antiviral therapy.

9 CONCLUSIONS

9.1 Development

9.1.1 *In vitro* NMR optimisation studies

Physical and NMR parameters have been optimised for the acquisition of reliable, high-resolution ^1H MAS NMR spectra from intact liver tissue, using the 11.7T JEOL system at the Hammersmith Hospital Campus, Imperial College London. MR parameters were set for subsequent experiments. Use of larger samples in the smallest cell able to contain them, was considered optimal. A spin speed of 4000Hz and acquisition temperature of 4°C were selected. Sample disruption increased spectral resolution but also increased spectral changes with time.

9.1.2 Phenotyping murine models of NAFLD

A novel approach to the investigation and classification of murine models of NAFLD on the basis of environmental, genetic and histopathological features using ^1H MAS NMR spectroscopy of intact liver tissue in conjunction with multivariate modelling techniques was established. Class membership was predicted with high accuracy and it was possible to distinguish reliably between samples, even where histological appearances were indistinguishable. Partial least squares discriminant analysis enabled the identification of biologically-relevant metabolites contributing to the differences between models.

Lipid content, measured using ^1H MAS NMR spectroscopy, was found to correlate with histological assessment of hepatocellular lipid droplets. Moreover, an exponential relationship between mouse mass and NMR-defined hepatic lipid load was established. Lipid composition (including indices of fatty acid polyunsaturation) differed according to dietary constituents and, independently, according to disease severity.

9.2 Translation

9.2.1 ^1H MAS NMR spectroscopy of liver tissue in patients with chronic hepatitis C

High resolution ^1H MAS NMR spectroscopy of liver biopsy samples was used to examine the metabolic profile in patients with chronic hepatitis C. Multivariate pattern-recognition analysis demonstrated that lipid and choline resonances accounted for much of the NMR spectral differences between groups: an increase in total lipid and a decrease in lipid polyunsaturation were observed with increasing hepatic fibrosis, while increased intrahepatic lipid was observed non-invasively in patients with genotype 3. This was the first study to apply these techniques to chronic hepatitis C and the largest such study of chronic liver diseases. Moreover, this is the first study using ^1H MAS NMR to demonstrate decreasing levels of hepatic polyunsaturated fatty acids with increasing degrees of liver fibrosis. On the basis of these studies, robust indices were proposed, which demonstrated potential to be translated to the *in vivo* setting.

9.2.2 *In vitro* data informs analysis and interpretation of MRS *in vivo*

Recently published work was criticised with respect to incorrect peak assignment. A physiologically-appropriate method to inform the analysis of *in vivo* MR spectra was proposed, whereby chemical shift information from ^1H MAS NMR spectra informs prior knowledge for curve-fitting of *in vivo* MR spectra.

9.2.3 Application of ^1H MRS to chronic hepatitis C *in vivo*

The indices examined *in vitro* were applied to a separate cohort of patients with chronic hepatitis C *in vivo*. The trends observed in the lipid composition indices *in vitro* were confirmed *in vivo*; specifically, that total lipid was higher in those with genotype 3, compared to those with genotype 1 and that PUI and CLR decreased with increasing hepatic fibrosis stage. Thus, ^1H MRS is a feasible method for the non-invasive assessment of lipid composition in hepatitis C-related chronic liver disease.

9.3 Evaluation

9.3.1 Comparison of non-invasive markers for the assessment of chronic hepatitis C

Two imaging-based markers (TE and HTT) and two serum markers (APRI and ELF) were compared with histological assessment of liver biopsy in patients with hepatitis C, both head-to-head and in combination, according to increasing cost and complexity. Individually, TE and ELF performed best for the prediction of fibrosis stage. In combination, APRI with either TE or ELF was optimal. Addition of further markers did not improve the prediction of fibrosis stage significantly. HTT performed least well for the prediction of fibrosis. Neither HTT, nor ELF had previously been evaluated in the context of other non-invasive markers of chronic liver disease.

9.3.2 Evaluation of ^1H MRS-derived indices compared to other markers

As the ^1H MRS-derived indices correlated with fibrosis stage, their ability to diagnose cirrhosis or predict fibrosis stage was compared to that of APRI and TE. Although both PUI and CLR predicted fibrosis stage, they performed less well than either APRI or TE individually. However, in combination with APRI or TE, they contributed significantly to the regression model to predict fibrosis, demonstrating that these indices are complementary to other markers when used in combination.

9.3.3 ^1H MRS in the assessment of chronic liver disease

In conclusion, the analysis of lipid composition is of pathogenic importance in chronic liver disease and the non-invasive assessment of hepatic lipid composition using ^1H MRS provides a novel perspective.

10 PUBLICATIONS

Listed below are articles related to the work contained in this thesis, which have been published or accepted for publication by the 30th September 2008:

10.1 Original research

Cobbold JFL, Anstee QM, Goldin RD, Williams HR, Matthews HT, North BV, Absalom N, Thomas HC, Thursz MR, Cox RD, Taylor-Robinson SD and Cox IJ. Phenotyping murine models of NAFLD through metabolic profiling of intact liver tissue. *Clinical Science* 2008 Aug 12; epub ahead of print

Morin SHX*, **Cobbold JFL***, Lim AKP, Eliahoo J, Thomas EL, Mehta SR, Durighel G, Fitzpatrick J, Bell JD and Taylor-Robinson SD. Incidental findings in healthy control research subjects using whole-body MRI. *European Journal of Radiology* 2008 Sep 23; epub ahead of print

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10.2 Other publications

Cobbold JFL, Taylor-Robinson SD and Cox IJ. *In vitro* ¹H MR spectroscopy of liver tissue informs *in vivo* hepatic ¹H MR spectroscopy studies. Correspondence. *Hepatology* 2008; 48: 1016-1017

Cobbold JFL and Taylor-Robinson SD. Liver stiffness values in healthy subjects: implications for clinical practice. *Journal of Hepatology* 2008; 48: 529-531

Cobbold JFL and Taylor-Robinson SD. Transient elastography in acute hepatitis: all that's stiff is not fibrosis. *Hepatology* 2008; 47:370-372

Cobbold JFL, Morin S and Taylor-Robinson SD. Transient elastography (FibroScan) for the assessment of chronic liver disease: ready for the clinic? *World J Gastroenterol.* 2007; 13(36): 4791-4797

Cobbold JFL, Wylezinska M, Cunningham C, Crossey M, Thomas H, Patel N, Cox J and Taylor-Robinson S. Non-invasive evaluation of hepatic fibrosis using magnetic resonance and ultrasound techniques. Letter. Gut. 2006; 55: 1670

Cox I J, Sharif A, **Cobbold JFL**, Thomas HC and Taylor-Robinson SD. Current and future applications of *in vitro* magnetic resonance spectroscopy in hepatobiliary disease. World J Gastroenterol. 2006;14; 12(30):4773-8

10.3 Published abstracts

Cobbold JF, Patel N, Goldin RD *et al.* Early haemodynamic responses to Interferon therapy in patients with chronic liver disease using contrast-enhanced ultrasound: regulation by hepatic stellate cells? Journal of Hepatology. 2008; 48:S271

Cobbold JF, Chinthapalli S, Anstee QM *et al.* Dietary constituents in mice are reflected in the hepatic lipid profile as assessed by carbon-13 magic angle spinning magnetic resonance spectroscopy. Hepatology 2007; 46:S1:1185

Cobbold JF, Anstee QM, Goldin RD *et al.* Can disease progression in non-alcoholic fatty liver disease be predicted by metabolic biomarkers of lipid accumulation and peroxidation? Journal of Hepatology. 2007; 46:S265

10.4 Other presentations

JFL Cobbold, JH Patel, SD Taylor-Robinson *et al.* British Association for the Study of the Liver (BASL) 2008: Polyunsaturation of fatty acids decreases with increasing stage of fibrosis in liver tissue from patients with chronic hepatitis C. Edinburgh, UK. 10-12 September 2008.

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