

**Prevalence, risk factors and sequelae of non-alcoholic fatty  
liver disease in Type 2 diabetes**

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## Dedication

For David.

## Declaration

1. This thesis was composed by me
2. The research presented was carried out within the Edinburgh Type 2 Diabetes Study, a large study in which many people have been involved. I had the responsibility of setting up the Year 1 “liver” study, the data from which is presented here. The basic study design was in place prior to my involvement. My role involved obtaining ethical approval for the study, liaising with specialists in the field of diabetology, public health, hepatology and radiology to formalise the study design, writing the study protocol, running the daily research clinics, dealing with the results and managing the data. The studies presented here were primarily analysed and written by me.
3. I acknowledge the following contributions
  - Drs Mark Strachan, Jackie Price and Rebecca Reynolds, along with the Edinburgh Type 2 Diabetes Study Investigators, set up the baseline study and were involved in the study design of the Year 1 liver study
  - Professor Ian Marshall from the Scottish Funding Council Brain Imaging Research Centre, Western General Hospital, Edinburgh designed the MRS sequences used in Chapter 5 and with Dr Calum Gray and their colleagues from the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh analysed the images.
  - Drs Stephen Glancy and Elisa Perry, and sonographer Lisa Nee from the Department of Radiology, Western General Hospital, Edinburgh contributed to the ultrasound protocol used throughout the study, and analysed the ultrasound images.
  - Professor Ramzi Ajjan and his team from the LIGHT Laboratories, Leeds designed the methodology for determination of clot formation and lysis dynamics and analysed the blood samples for this part of the study.
4. This thesis has not been submitted for any other degree or professional qualification

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Rachel MacLeod Williamson

Date: 14th June 2013

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# THE UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS

Regulation 3.1.14 of the Postgraduate Assessment Regulations for Research Degrees refers  
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Type 2 diabetes is an established risk factor for non-alcoholic fatty liver disease (NAFLD). Despite this, there are few data investigating NAFLD within large populations of people with Type 2 diabetes. In the initial chapters of this thesis NAFLD is defined and the pathological processes linking it to Type 2 diabetes are described. The epidemiological evidence that underpins current understanding of prevalence, risk factors and progression of the condition is reviewed in detail. Subsequent chapters describe research undertaken within the Edinburgh Type 2 Diabetes Study on the epidemiology of NAFLD in this population.

The prevalence of NAFLD was determined within 939 subjects, aged 61 – 76 years, with well-characterised Type 2 diabetes. NAFLD was defined by the presence of ultrasound-diagnosed steatosis with detailed exclusion of secondary causes for liver disease. Ultrasound gradings were validated in a subgroup of 58 participants using <sup>1</sup>H magnetic resonance spectroscopy, the non-invasive gold-standard method for hepatic lipid quantification, and intra- and inter-observer variability in ultrasound grading was calculated. The final prevalence of NAFLD, 42.6%, was higher than in the general population, but lower than in the few studies that have been performed in populations with Type 2 diabetes. It is likely that this was due to comprehensive screening for secondary causes of liver disease and validation of the ultrasound measure which resulted in re-categorisation as “normal” subjects initially graded as having mild steatosis. Independent predictors of NAFLD were diabetes variables or components of the metabolic syndrome.

The utility of conventional “liver function tests” in detecting hepatic steatosis and NAFLD was examined. Although liver enzyme levels (alanine aminotransferase, aspartate aminotransferase and gamma-glutamyltransferase) were significantly higher in participants with hepatic steatosis compared with those with normal liver on ultrasound, values remained within the normal range in the majority of cases. The negative predictive values of normal levels were therefore low, calling into question their utility as screening tests for liver disease in the diabetic population.

The prevalence of advanced liver disease had not previously been studied in a population with Type 2 diabetes, and was investigated in our population using surrogate markers of hepatic fibrosis. Hyaluronic acid (HA) levels were significantly elevated in 5.7% of subjects and 0.4% had ultrasound-diagnosed cirrhosis. Hepatocellular carcinoma was detected in 0.2% of participants. Liver enzyme markers were poorly predictive of high HA levels. Clinical risk scores identified a large proportion of subjects as potentially having severe fibrosis, but these scores have not previously been validated in populations with Type 2 diabetes and therefore have to be interpreted with caution.

NAFLD has previously been shown to be predictive of cardiovascular disease in the context of Type 2 diabetes, but mechanisms underlying this have not been fully elucidated. In the current study the association of hepatic steatosis and NAFLD with non-classical cardiovascular risk factors – clot formation and lysis dynamics, prothrombotic mediators and markers of inflammation – was investigated. NAFLD was significantly associated with a longer clot lysis time and higher levels of complement C3 and plasminogen activator inhibitor type 1. Following adjustment for these prothrombotic compounds, the association of NAFLD and clot lysis time was lost, and it is therefore hypothesised that NAFLD may influence clot lysis time via increased production of these mediators.

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## List of abbreviations

ALT	Alanine aminotransferase
AlkP	Alkaline phosphatase
ANA	Antinuclear antibody
apoB100	Apolipoprotein B 100
AST	Aspartate aminotransferase
AUC	Area under the curve
BMI	Body mass index
BP	Blood pressure
CIMT	Carotid intima media thickness
CT	Computerised tomography
CTGF	Connective tissue growth factor
ELF	European liver fibrosis panel
ET2DS	Edinburgh Type 2 Diabetes Study
FF	Fat fraction
FFA	Free fatty acids
GGT	Gamma glutamyltransferase
HA	Hyaluronic acid
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HOMA IR	Homeostatic model assessment of insulin resistance
IL-6	Interleukin-6
IRS	Insulin receptor substrate
ISD	Information and services division
LDL	Low density lipoprotein
LDR	Lothian Diabetes Register

LFT(s)	Liver function test(s)
MRS	Magnetic resonance spectroscopy
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acids
NF- $\kappa$ B	Nuclear factor kappa-light-chain enhancer of activated B cells
NHANES	National Health and Nutrition Examination Study
NPV	Negative predictive value
PAI-1	Plasminogen activator inhibitor type 1
PPV	Positive predictive value
PSR	Platelet count:spleen diameter ratio
P3NP	Aminoterminal peptide of procollagen III
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
TIMP-1	Tissue inhibitor of matrix metalloproteinase 1
TNF $\alpha$	Tumour necrosis factor $\alpha$
TNFR	Tumour necrosis factor $\alpha$ receptor
tPA	Tissue plasminogen activator
VLDL	Very low density lipoprotein
WC	Waist circumference
WHR	Waist:hip ratio

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# Diagnosis and pathophysiology of non-alcoholic fatty liver disease (NAFLD)

## 1.1 Introduction

Liver disease is a significant health and economic problem both in the United Kingdom (UK), where it is the fifth most common cause of death, and worldwide(1). Historically, two aetiologies – alcoholic liver disease and chronic viral hepatitis - have been recognised as making the largest contribution to this burden. In the last three decades, however, a third aetiology has been increasingly recognised in the form of the obesity-related non-alcoholic fatty liver disease (NAFLD)(2).

Since its initial description in 1980 by Ludwig and colleagues(3), NAFLD has been acknowledged as the most common liver-related medical condition in Westernised countries, affecting as much as one third of the general population(4;5). Its close relationship with central obesity, insulin resistance and Type 2 diabetes has fostered the proposal that it is the hepatic component of the metabolic syndrome(6), and studies have suggested that it is an independent risk factor for cardiovascular disease(7). Furthermore, NAFLD is a documented cause of cirrhosis and hepatocellular carcinoma (HCC), and accounts for over 10% of patients on the waiting list for liver transplantation in the UK(8).

Despite the body of research on the long-term outcome of NAFLD and the persuasive evidence that the combination of NAFLD and diabetes can be associated with a relatively poor prognosis there is no consensus on screening for or managing this condition within the context of Type 2 diabetes. Neither the National Institute for Clinical Excellence guidelines for the management of Type 2 diabetes, nor guidelines with a similar scope produced by the Scottish Intercollegiate Guidelines Network, give guidance on liver disease within the context of Type 2 diabetes. Moreover, there is evidence of variation between clinicians from

different specialties in their response to markers of liver disease in such patients(9). This is likely to reflect a number of features of the evidence available, in particular the relative paucity of data from otherwise unselected populations of patients with Type 2 diabetes, the absence of an accurate and simple test for use within the diabetes clinic room to diagnose and stage NAFLD, and the shortage of effective and available treatments. In keeping with this, the American Association for the Study of Liver Diseases has identified as necessary further research into NAFLD in the context of Type 2 diabetes(10).

In this and following chapters the body of literature on the pathogenesis and epidemiology of NAFLD, with particular respect to its incontrovertible link with Type 2 diabetes, will be explored and discussed.

## 1.2 Definition of NAFLD

NAFLD encompasses a spectrum of disease, from simple steatosis (“fatty” change) to steatohepatitis (an inflammatory state), fibrosis and cirrhosis. The pathological appearances on histological examination are identical to those found in alcoholic liver disease, a factor that may have contributed to delay in the recognition of NAFLD as a specific disease entity. The original paper to describe NAFLD actually defined non-alcoholic steatohepatitis (NASH) in a group of 20 patients who had biopsy findings mimicking alcoholic hepatitis, but in the absence of an alcohol precipitant(3). Over time, the definition of the condition has broadened to include people with hepatic steatosis alone without a significant inflammatory or fibrotic component (also termed non-alcoholic fatty liver (NAFL)). Furthermore, the previously termed “cryptogenic cirrhosis” has been recognised in many cases to represent the most severe end of the NAFLD spectrum(11;12). Liver fat accumulation accounting for over 5-10% of liver weight in the absence of alcohol excess or other causes of liver disease is characteristic of both simple steatosis and NASH(10). Practically, this has been interpreted as over 5% of parenchymal involvement by steatosis on liver biopsy(13). It is however well recognised that steatosis can regress as fibrosis and cirrhosis develop(14).

It is recognised that a number of conditions, over and above alcohol excess, can be associated with pathological findings suggestive of NAFLD. These have been classified into primary and secondary NAFLD, and for the purposes of this review I will focus on “primary” NAFLD, i.e. that which is associated with insulin resistance and features of the metabolic syndrome, as outlined below. “Secondary” causes of NAFLD are varied, and include particular medications, disorders of lipid metabolism (including abetalipoproteinaemia), mitochondrialopathies, severe weight loss following bariatric surgery (particularly jejunoileal bypass), total parenteral nutrition and exposure to industrial solvents(10;15-17).

Although the initial description of NAFLD was based on clinicopathological findings, the majority of diagnoses, particularly in population-based studies, are now made by non-invasive means(5). A working definition of NAFLD as being the presence of hepatic steatosis on imaging in the absence of a secondary cause for this has been adopted and used in a number of studies(18-20), although limitations of this approach have been

recognised(16;21). In particular, this classification has designated NAFLD as a disease of exclusion, rather than a condition that is defined according to its aetiology. Aside from intellectual considerations, this has practical implications in that NAFLD can technically only be diagnosed in the absence of other hepatic diseases despite indications that NAFLD may co-exist with other liver disease and indeed that the presence of NAFLD may influence the natural history of other liver pathology(22).



## 1.3 Pathophysiology of NAFLD

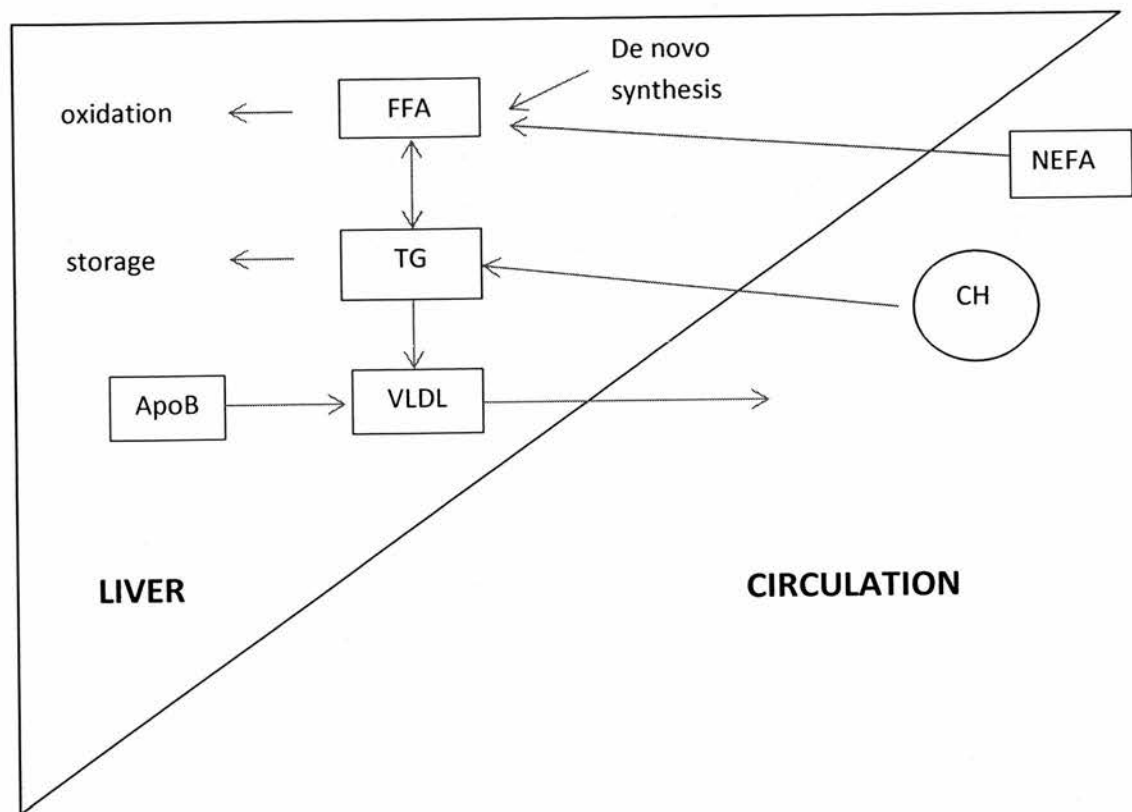
### 1.3.1 Lipid metabolism in health

The liver plays a significant role in normal lipid metabolism and an understanding of this is helpful in elucidating the pathophysiological processes of NAFLD. The body's fat stores are in the form of triglyceride, each molecule of which comprises a "spine" of glycerol esterified to three fatty acids(23). Although most triglyceride is stored within adipose tissue, there can also be deposition in organs such as the liver and muscle. Hepatic triglyceride originates both from dietary sources and from synthesis within hepatocytes. Dietary lipid is transported via peripheral tissues such as muscle and adipose tissue and arrives at the liver within a chylomicron remnant containing triglyceride and cholesterol ester. Synthesis of triglyceride within the liver utilises both fatty acids that are made *de novo*, and non-esterified (free) fatty acids (NEFA) removed from the circulating pool. Export of triglyceride from hepatocytes occurs within very low density lipoprotein (VLDL), a complex of protein, lipids and phospholipids that contains apolipoprotein B100 (apoB100). These pathways are summarised below in Figure 1.1.

It is clear that the maintenance of a normal liver depends on a complex balance between hepatic triglyceride synthesis and storage on the one hand, and utilization and export on the other. This balance can be disturbed by factors both intrinsic and extrinsic to the liver, resulting in triglyceride accumulation and hepatic steatosis. Such perturbations will be considered in the next section.

Figure 1.1

Role of the liver in triglyceride metabolism. The main factors governing triglyceride content of the liver are shown.



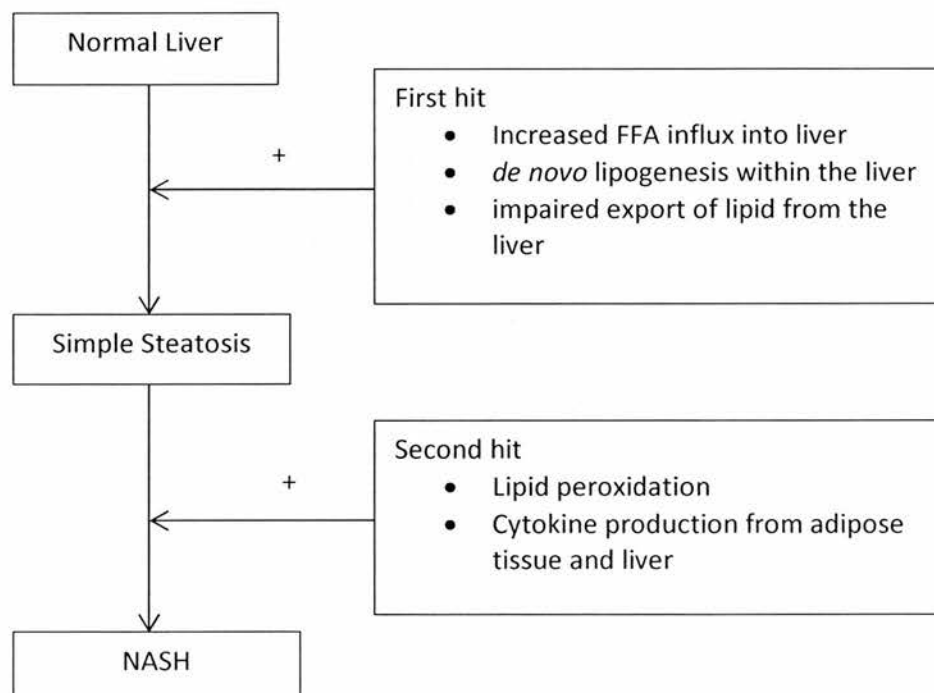
ApoB, apolipoprotein B100; CH, chylomicron remnant containing triglyceride; FFA, free fatty acids; NEFA, non-esterified fatty acids; TG, triglycerides; VLDL, very low density lipoprotein

### 1.3.2 Pathophysiology of NAFLD

There is no single unifying model to describe the events in the establishment of NAFLD and its progression to inflammatory and fibrotic liver disease. Nevertheless, the “two-hit hypothesis”, proposed by Day & James in 1998(24), has been the most influential and prevailing framework in explaining the pathogenesis. Summarised in Figure 1.2, this model suggests that hepatic steatosis (the “first hit”) is the initial event and a necessary component in the development of NASH, but is not itself sufficient to promote this advancement. A “second hit” is then required to stimulate the progression to more advanced liver disease. This theory is attractive as it goes some way towards explaining why only a subgroup of people with NAFLD go on to develop NASH, fibrosis and cirrhosis.

Figure 1.2

The “two hit” hypothesis, which explains the variable progression of NAFLD to NASH(24)



### *First hit*

Although the pathophysiology of NAFLD is incompletely understood, it is accepted that excess triglyceride accumulation within hepatocytes is a central event. Three pathways, as demonstrated above in Figure 1.2, have been described as promoting this state: (1) increased free fatty acid (FFA) influx into the liver; (2) de novo lipogenesis within the liver; and (3) impaired export of lipid from the liver. A fourth possible mechanism, reduced FFA oxidation, has not been shown to play a role(25). Differing degrees of insulin resistance in different tissues appear to play an important role in increasing the burden of hepatic fat.

#### (1) Increased FFA influx into the liver

Insulin resistance in peripheral tissues has been postulated to be the initial event in the pathogenesis of hepatic steatosis(26). Physiologically, insulin acts to suppress lipolysis in adipose tissue and when its actions there are inhibited, as can occur in the context of excessive fat accumulation, there is dysregulation of lipolysis that increases the supply of FFA to the liver. One study which used isotope methodology to quantify relative contributions to hepatic triglyceride accumulation in nine patients with NAFLD who underwent a medically-indicated liver biopsy, found that over half (59%) of hepatic triglyceride arose from NEFA from adipose tissue(27). The role of FFA flux from adipose tissue to liver is further supported by a study of 18 individuals (6 NASH, 6 steatosis alone, 6 controls) in which the percentage suppression in appearance of FFA and glycerol in the circulation (a measure of lipolysis) in response to insulin infusion was significantly less in the steatosis and NASH groups compared with controls(28). This finding of a reduction in insulin-mediated suppression of lipolysis in people with NAFLD has been mirrored elsewhere(6;29). There is significant, albeit indirect, evidence that visceral adiposity (rather than peripheral adipose tissue) may have particular significance in the development of hepatic steatosis. It has been shown that people with NAFLD may have an increased waist:hip ratio (WHR) and waist circumference (WC, measures of abdominal adiposity), relative to body mass index (BMI)(6;29), and the relative preponderance of visceral fat and its independent role in predicting liver fat has been confirmed on magnetic resonance imaging (MRI) studies that have quantified adiposity directly(29-31). It has also been proposed that visceral adipose tissue is more insulin resistant and prone to lipolysis than subcutaneous adipose tissue(32). From an anatomical perspective there is a direct link

between abdominal fat and the liver and any FFA released from central adipose stores are delivered directly to the liver via portal venous drainage.

## (2) de novo lipogenesis within the liver

Hepatic production of fatty acids appears to contribute to a relatively minor proportion of the triglyceride that is synthesised within and secreted from the liver in health, but its contribution may increase in NAFLD. Evidence originates from two isotope studies, one of which is outlined above(27). In this study of patients with NAFLD, 26% of triglyceride on liver biopsy had been produced by de novo lipogenesis. This was a smaller proportion than that produced from NEFAs from adipose tissue, but a higher percentage than that produced from dietary fatty acids. In a second study, hepatic lipogenesis was measured using the stable isotope deuterated water, which is incorporated into newly synthesised fatty acids but is not present in fatty acids or triglyceride from other sources(33;34). The calculated contribution of lipogenesis to hepatic triglyceride *secretion* was three times higher in the five NAFLD subjects than in controls (14.9% vs 4.6% respectively); it is expected that there would be a similar contribution to hepatic triglyceride *content*. It has been hypothesised that the increase in lipogenesis is driven by high insulin levels and mediated via the transcription factor sterol regulatory element binding protein (SREBP-1c)(35); this theory assumes that this hepatic pathway remains sensitive to the effects of insulin, in the presence of peripheral resistance.

## (3) Impaired export of lipid from the liver

The assembly of VLDL, in the process of triglyceride export from the liver, is a complex process and there several points in the pathway that are susceptible to impairment. One area of interest is the synthesis of apoB100, a rate-limiting step in VLDL formation. Rate of synthesis has been shown to be significantly lower in one study of patients with NASH (31.5 mg/kg/day) compared to BMI-matched (115.2 mg/kg/day) and lean (82.3 mg/kg/day) controls, and this appeared to be an effect specifically on apoB100 rather than a generalised effect on all protein synthesis(36). It is, however, impossible to be sure in this cross-sectional study that the results seen are not a consequence rather than a cause of NASH. Furthermore, results from another report(37) have suggested that there is a significant difference in apoB100 synthesis rates between simple steatosis and NASH and this calls into question the theory that impaired export of VLDL by this mechanism is the primary event in the

pathogenesis of NAFLD (as opposed to a step in its progression to NASH). In an additional study hepatic steatosis was associated with an increase in apoB100 secretion within VLDL(38). Assuming that inhibition of apoB100 formation does play a role, it seems likely that hyperinsulinaemia is once again a key step in this pathway(39), but results from studies examining this have been mixed(40).

### *Second hit*

The steatotic liver is vulnerable to further insults which promote hepatocyte injury, inflammation and fibrosis. It has been proposed that triglyceride storage (rather than being hepatotoxic in itself) is a marker of increased exposure to potentially-toxic FFA, and that simple steatosis progresses to NASH when mechanisms that protect hepatocytes from FFA-mediated lipotoxicity are exhausted(14). Both oxidative stress and increased cytokine production have been proposed as mediators in the pathogenesis of NASH and subsequent fibrosis.

#### (1) Lipid peroxidation

Lipid peroxidation induced by reactive oxygen species (ROS) has previously been shown to be capable of causing the pathological features typical of NASH-associated inflammation and fibrosis - including stimulation of neutrophil chemotaxis, formation of Mallory bodies, activation of hepatic stellate cells (which are key players in fibrogenesis), and hepatocyte necrosis and apoptosis(41). One possible mechanism of ROS generation is mitochondrial dysfunction. A study comparing liver biopsy specimens from patients with NASH, simple steatosis and controls found abnormal mitochondrial morphology on electron microscopy in the samples from those with NASH(28); such abnormalities have previously been associated with an uncoupling of oxidation and phosphorylation, ROS production and subsequent lipid peroxidation. Mitochondrial abnormalities in the same study were paralleled by a higher level of mitochondrial fatty acid  $\beta$ -oxidation in these patients. Although the cross-sectional nature of this study precluded assessment of whether the mitochondrial abnormalities were a cause or an effect of NASH, it was hypothesised that clinically silent mitochondrial abnormalities present in a proportion of the normal population can be “unmasked” by the increase in FFA  $\beta$ -oxidation seen in hepatic steatosis, predisposing to inflammation.

A further proposed source of ROS in NAFLD is enhanced hepatic expression of cytochrome P450 2E1 (CYP2E1). This pathway was initially considered to be of possible relevance as hepatic CYP2E1 expression is increased in the context of alcoholic liver disease, and is associated with increased production of ROS in this patient group. A study examining biopsies from patients with NASH revealed a significant increase in CYP2E1 compared to controls, and a similar pattern of immunostaining to patients with alcoholic liver disease(42); this appeared to be an increase that was specific to CYP2E1 rather than a generalised increase in cytochrome P450 enzymes. As the visual pattern of increased CYP2E1 followed that of fat accumulation within hepatocytes, it can be hypothesised that the increase in CYP2E1 is driven by the lipid accumulation. Further support for a role of this pathway comes from a study that examined the activity of CYP2E1, as measured using the hydroxylation of chloroxazone, in patients with NASH versus age-, gender- and BMI-matched controls(43). In this study there was an increased activity of the enzyme in the context of NASH (with an increased rate of clearance of serum chloroxazone and an increased rate of appearance of urinary 6-hydroxy chloroxazone in this group). Although this pathway is clearly of interest, both of the above studies were of cross-sectional design and therefore not sufficient to imply a definite causal role of CYP2E1 in the pathogenesis of NASH.

## (2) Cytokines

The cross-sectional associations between NAFLD and inflammatory mediators are well-established but causal relationships have been more difficult to define. As roles in cytokine production have been ascertained for both visceral adipose tissue and the liver, one area of complexity is determining the source of circulating inflammatory molecules and establishing the pathological sequence of events. The evidence supporting a causal role for the pro-inflammatory cytokines tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-6 (IL-6) in the pathogenesis of NAFLD and its progression to NASH, along with evidence for their production in both adipose and hepatic tissue, is explored below. Adiponectin, a hepatoprotective cytokine, has also been shown to be significantly, but inversely, related to NAFLD and its progression, but will not be considered further here.

Attempts to confirm whether TNF $\alpha$  has a causative role in promoting progression of NAFLD have involved both animal and human studies. One approach has been to use mouse models

of NAFLD (including NAFLD-inducing diets or ob/ob mice) and examine the effect of TNF $\alpha$  inhibition with anti-TNF $\alpha$  antibodies on liver histology(44;45). In contrast to the controls, the liver biopsies of mice receiving anti-TNF antibodies showed markedly improved histology including reduced hepatic steatosis, inflammation and fibrosis. A further study compared mice genetically deficient in both TNF $\alpha$  receptors (TNFR knockout mice) with their wild-type counterparts after ingestion of NASH-inducing diet(46). Both TNFR-knockout and wild-type mice showed a significant increase in hepatic triglyceride levels after administration of the diet (compared to control mice fed an isocaloric diet), but this increase was significantly lower, and there was a markedly reduced amount of hepatic fibrosis, in the TNFR-knockout mice. The supportive evidence for a causative role of TNF $\alpha$  in NAFLD disease progression from animal studies has been mirrored by studies in humans that have examined the effect of TNF $\alpha$  inhibition and different TNF $\alpha$  polymorphisms on liver disease. Two small studies of patients with biopsy-proven NASH have examined the effects of a 6-12 month trial of pentoxifylline (a phosphodiesterase inhibitor that inhibits TNF $\alpha$  production)(47;48). In one case aminotransferase levels fell over the study period, but repeat biopsies were not performed, there was no control group and it was unclear if the effect on transaminases was independent of the significant reduction in BMI observed(47). In the second study, which again had no control group, 55% had a reduction in steatosis or lobular inflammation and 67% showed reduction in fibrosis on repeat biopsy(48). In genetic studies, attention has focussed on the relationship of biopsy findings within NAFLD to single nucleotide polymorphisms in the promotor region of the TNF $\alpha$  gene, whose polymorphic variant leads to a higher rate of transcription compared to the wild allele. Results have been variable, with some variant alleles associated with significantly higher rates of moderate/severe (vs mild) inflammation, NASH and presence of fibrosis than those with the wild allele(49;50), while in other cases trends failed to reach statistical significance(51). Although perhaps not as conclusive as the results from animal models, the above human studies also broadly support a causative role for TNF $\alpha$  in promoting NAFLD disease progression. Evidence for a role of IL-6 is less direct and there are no studies that have formally assessed the effect of IL-6 on liver histology. There is, however, evidence from murine studies that chronic exposure to IL-6 induces hepatic insulin resistance(52).

The role of different tissues, specifically adipose tissue and the liver, in producing TNF $\alpha$  and IL-6 has been explored in other studies. Significantly higher levels of TNF $\alpha$  secretion, and a trend towards higher levels of IL-6 secretion, from adipose tissue biopsy samples have been



observed in obese human subjects compared with lean controls(53;54). Following weight loss in obese subjects, significantly lower levels of TNF $\alpha$ -and IL-6 mRNA and protein expression within fat biopsy specimens was observed, compared with levels pre-weight loss(55;56). The increased production of cytokines from the expanded fat mass in humans with obesity has been postulated to be a consequence of relative adipose tissue hypoxia, caused by expansion of adipose tissue. It has been proposed that this is the initial event in a necroinflammatory process that promotes an increasing number of cytokine-producing resident macrophages that in turn perpetuate the cycle of inflammation(57).

A higher production of TNF $\alpha$  and IL-6 in liver tissue in patients with NASH has also been described. In patients undergoing bariatric surgery for obesity, the mRNA expression of TNF $\alpha$  and its receptor was significantly higher in the liver biopsy samples of patients with NASH compared to those with normal liver histology or simple steatosis(58). When patients with NASH were further subdivided according to the presence or absence of fibrosis, there was a grade-dependent increase in TNF $\alpha$  mRNA expression through the fibrosis groups; a close correlation between mRNA expression in liver and adipose tissue was also observed. In a study performed on liver tissue from humans with biopsy-proven NASH, simple steatosis or normal liver, hepatic IL-6 expression, as assessed by immunohistochemistry using a monoclonal antibody, was significantly higher in NASH patients compared with simple steatosis patients with a further significant difference between steatosis patients and controls(59). Furthermore, IL-6 expression was significantly correlated with stage of fibrosis, and was higher in patients with fibrosis compared to those without. These findings have been mirrored in animal studies(60). Interestingly, in a study examining the effect of anti-TNF $\alpha$  antibodies on liver pathology in mice, the hepatocyte expression of TNF $\alpha$ -mRNA was reduced in those mice receiving the antibody, providing evidence for the circular relationship of TNF $\alpha$  expression and inflammation(44).

To outline the cellular mechanisms of TNF $\alpha$  and IL-6 production and action in detail is beyond the scope of this review. A couple of points, however, deserve mention with the particular aim of relating cytokine pathophysiology to the other pathogenic processes outlined above. There is evidence that lipid accumulation within hepatocytes, along with oxidative stress, is a stimulus for hepatic cytokine production via activation of the nuclear factor kappa-light-chain enhancer of activated B cells (NF- $\kappa$ B) pathway (the inflammatory

“master-switch”) which in turn promotes production of cytokines including TNF $\alpha$  and IL-6(61). In this way hepatic steatosis *per se* can predispose to the more inflammatory NASH. Downstream, molecular pathways of action of cytokines have been shown to enhance insulin resistance (via Jun N-terminal kinase activity, a TNF $\alpha$ -dependent enzyme that causes insulin resistance by increasing the serine phosphorylation of insulin receptor substrate (IRS)-1 and -2) and activate Kupffer cells (liver-specific macrophages) that in turn contribute to the inflammatory milieu(62). Thus, inflammatory cytokines contribute to hepatic insulin resistance that in turn exacerbates whole body insulin resistance.

## **1.4 Diagnostic tools for NAFLD**

Although symptoms of NAFLD (in particular right upper quadrant discomfort and fatigue) are recognised, the majority of patients are asymptomatic until advanced liver disease develops(15). As a result, most ascertained cases are discovered incidentally or as a result of biochemical screening investigations. There is legitimate concern regarding the accuracy of liver enzymes in this role, and it is clear that the majority of cases are unrecognised(63;64). Definitive diagnosis involves biopsy but in reality this is undertaken in a minority of cases, with the remainder of diagnoses, particularly in population-based research studies, being made on imaging. The evidence underpinning the use of these investigative tools is explored in this chapter.

### **1.4.1 Biopsy**

Histological examination of liver tissue, in combination with careful ascertainment for secondary causes of hepatic damage, is acknowledged as the gold standard method of diagnosing and staging NAFLD. An understanding of the functional anatomy of the liver is important in understanding the terminology used. On a microscopic level, the liver is made up of multiple small lobules at the mid-point of which is the central vein(65). Radiating out from the central vein are sinusoids separated from each other by hepatocytes and peripheral portal tracts. Hepatocytes are divided functionally into zones. Zone 1 refers to the peri-portal area, which is closest to the hepatic artery (and portal vein) and therefore receives the most oxygenated blood. Zone 3 is located around the central vein and therefore receives the poorest oxygenation and is most sensitive to ischaemic injury. Zone 2 is intermediate between zones 1 and 3.

Several grading/staging systems have been used to histologically define NAFLD and these are outlined below.

*Brunt et al.(66)*

- Steatosis graded 1 - 3
  - Steatosis  $\leq$  33% parenchymal involvement
  - Steatosis 33 – 66% parenchymal involvement
  - Steatosis  $\geq$  66% parenchymal involvement
  
- Necroinflammatory activity graded 0 – 3
  - 0 - none
  - 1 - mild, occasional ballooned (dead) hepatocytes, mild lobular inflammation, no or mild portal inflammation
  - 2 - moderate, obvious hepatocyte ballooning, mild lobular inflammation, mild to moderate portal inflammation
  - 3 - severe, marked hepatocyte ballooning, scattered lobular inflammation, mild to moderate portal inflammation
  
- Fibrosis staged 0 – 4
  - 0 – none
  - 1 - zone 3 perisinusoidal fibrosis
  - 2 - as 1 plus portal fibrosis
  - 3 - as 2 with bridging (central to portal or central to central) fibrosis
  - 4 - cirrhosis

*Matteoni et al. (67)*

- NAFLD Type 1 – steatosis alone
- NAFLD Type 2 – steatosis with lobular inflammation only
- NAFLD Type 3 – steatosis with hepatocellular ballooning (NASH)
- NAFLD Type 4 – steatosis with Mallory hyaline (a protein inclusion found in the cytoplasm of hepatocytes) or fibrosis (NASH)

*NASH Clinical Research Network Scoring System(13)*

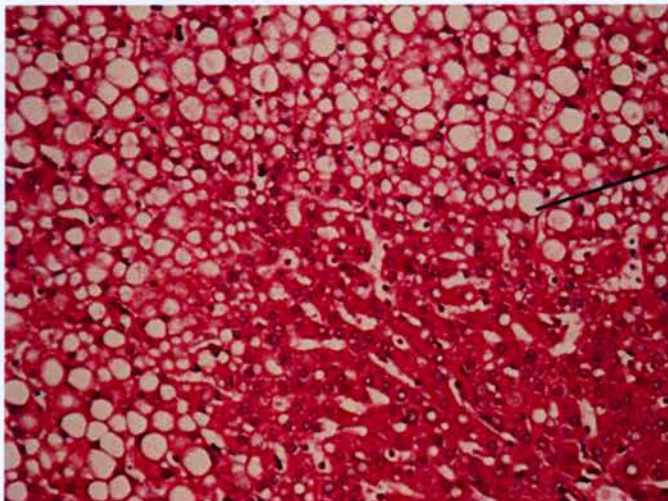
- Steatosis scored 0 – 3 on the basis of percentage of parenchymal involvement (< 5%, 5 – 33%, 33 – 66%, > 66%), location and presence of microvesicular steatosis
- Inflammation and liver cell injury scored on the basis of features including lobular and portal inflammation and hepatocyte ballooning
- Fibrosis staged 0 – 4 (0, no fibrosis; 1, perisinusoidal or periportal; 2 perisinusoidal and portal/periportal; 3, bridging fibrosis; 4, cirrhosis)

Examples of biopsy specimens demonstrating simple steatosis and NASH are shown below (Figure 1.3)

*Figure 1.3*

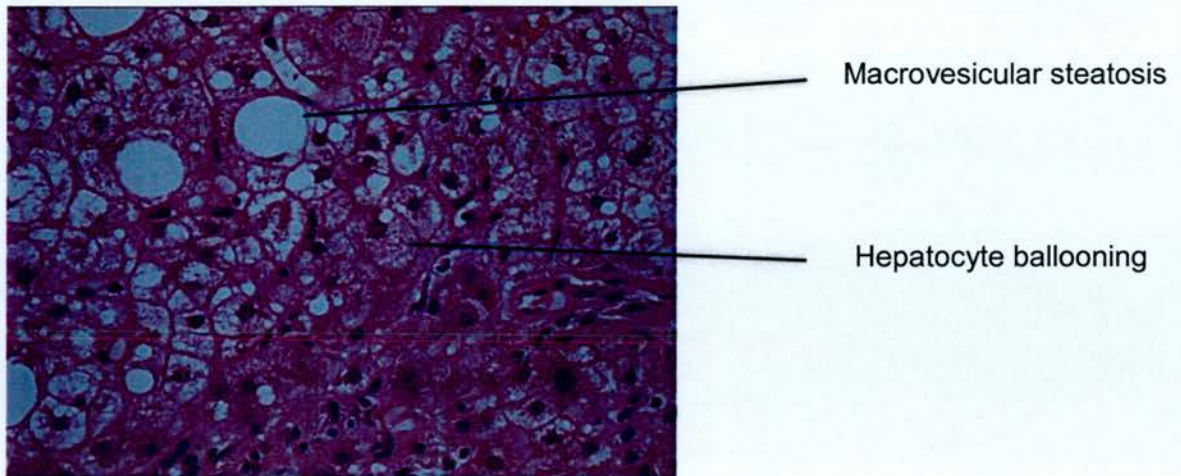
Biopsy slides of (a) NAFL (simple steatosis) and (b) NASH (*slides courtesy of Dr Chris Bellamy*)

(a)



Macrovesicular steatosis

(b)



Although there have been encouraging results from studies that have attempted to non-invasively identify more advanced NAFLD, biopsy continues to play an important role in cross-sectional and longitudinal studies. Self-evident limitations include its invasive nature and the, albeit small, risk of side-effects including haemorrhage. Furthermore, a percutaneous biopsy sample is representative of 1/50000 of the total mass of the liver and there is evidence that sampling error can lead to misclassification, particularly in staging of NASH and fibrosis(68;69). Studies also suggest that intra- and inter-observer agreement in grading is imperfect. Inter-observer agreement has been as high as 0.85 and 0.84 respectively for stage of fibrosis, and 0.83 and 0.79 for steatosis, but much lower for some features of inflammation, such as lobular and portal inflammation (0.60 and 0.45, and 0.55 and 0.45 respectively), and in one study the agreement on the diagnosis of NASH was only 0.61(13).

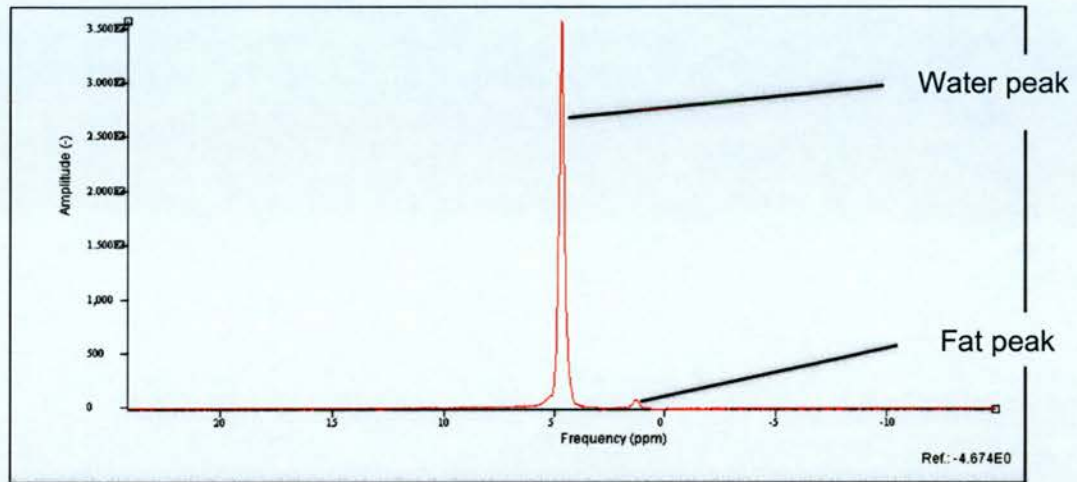
#### **1.4.2 Imaging modalities – magnetic resonance spectroscopy**

<sup>1</sup>H magnetic resonance spectroscopy (MRS), widely recognised as the non-invasive gold standard for measurement of hepatic fat, has a high correlation with liver fat concentration on biopsy, of the order of 0.7 - 0.9(70-72). MRS produces a spectrum of the different resonances of protons that are embedded in different chemical bonds, relying on the fact that protons within different bonds are exposed to differing patterns of nuclei and electrons that in turn cause small magnetic field perturbations which affect received frequencies of protons. Hepatic MRS uses the ratio of the proton peak from fat to the proton peak from water to determine the fat fraction (FF). Examples of the spectra seen are shown in Figure 1.4.

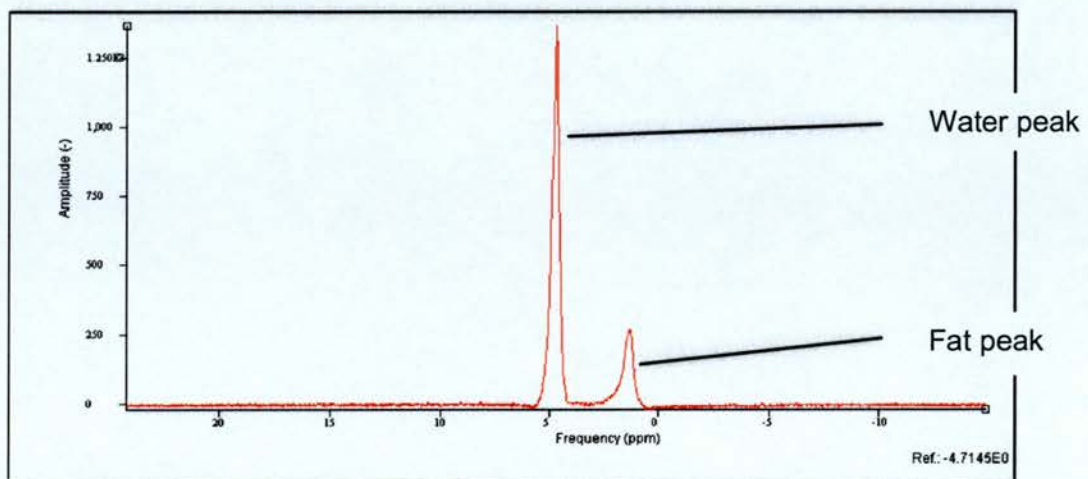
Figure 1.4

MRS spectra from (a) a normal liver and (b) a liver with significant steatosis (FF 22%). Note the larger fat peak in (b).

(a)



(b)



Different “normal” values for hepatic FF on magnetic resonance imaging have been reported previously(63;73;74). In an early study, 28 healthy volunteers underwent MR scanning by a modified Dixon gradient echo technique. All had a MR hepatic FF under 9% and this was later considered as being the upper limit of normal(73;74). In the same study seven subjects with cystic fibrosis underwent both MRI scanning and liver biopsy: two subjects with no steatosis on biopsy had a FF on MRI of < 9%; five subjects with mild to severe steatosis had a FF of > 9%. Later studies, however, have suggested that a lower cut-off of FF is a more realistic indication of normality, and the Dallas Heart Study was a significant study in this regard(63). In this population, normal hepatic FF was defined as the 95<sup>th</sup> percentile of hepatic triglyceride content in a group of 345 participants who were selected to be low risk for hepatic steatosis (normal BMI, glucose tolerance and liver enzymes, and non-excessive alcohol use). The value corresponding to the 95<sup>th</sup> percentile (expressed as grams triglyceride per 100 grams wet liver tissue) was 5.5%, which in turn corresponded to an MRS FF of 6.1%(70). A prospective study of 161 subjects, with a rate of hepatic steatosis > 5% on biopsy of 44%, found sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of MRS (hepatic triglyceride content cut-off of 5.7%) to be 80%, 80%, 71% and 87% respectively(75). These results were mirrored or bettered in other smaller studies(72;76;77), and in a meta-analysis which found values for sensitivity and specificity to be 88.5% and 92% respectively(78).

MRS has been used in one large scale study of over 2000 participants but, in general, studies have been small, presumably limited by expense. Advantages that might promote its use in future (longitudinal) studies are that it allows accurate quantification of steatosis while avoiding serial liver biopsies. Weaknesses include “within liver” variability in results(79) and the fact that it has no role in grading more advanced liver disease such as NASH.

### **1.4.3 Imaging modalities - ultrasound**

Ultrasound imaging is an established imaging modality in the diagnosis of hepatic steatosis, both clinically and in large-scale studies. Although several grading systems have been proposed for the assessment of hepatic steatosis using ultrasound, no consensus has been achieved(16;80-83). Detection of hepatic fat is based on well-established characteristics including an echogenic parenchyma (especially in relation to the right kidney), posterior attenuation (posterior darkness and loss of definition of the diaphragm) of the ultrasound

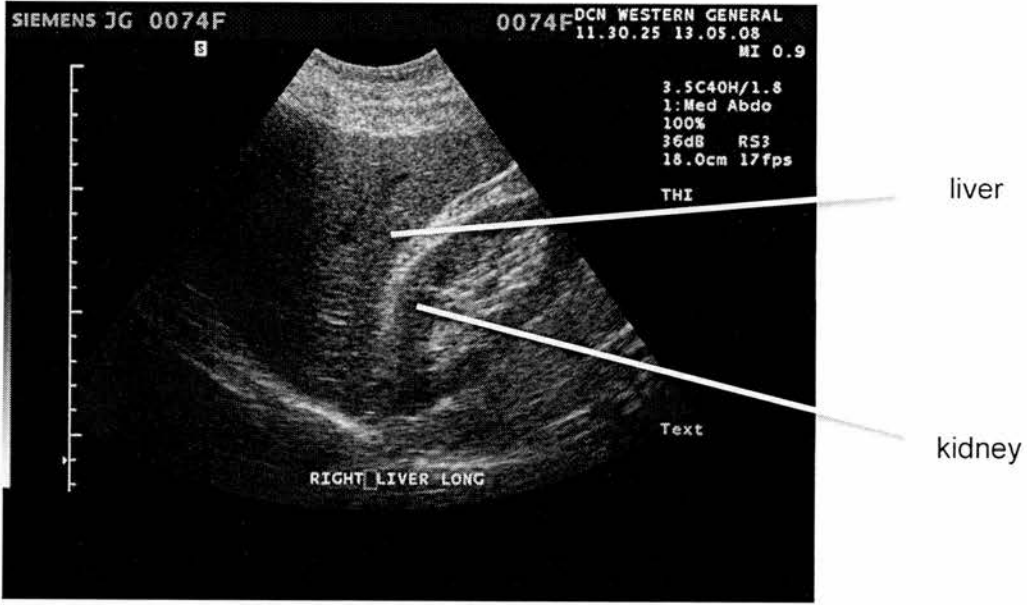


beam as it passes through the liver, and areas of focal fatty sparing(16;82-85). Examples of ultrasound images from normal and steatotic livers are shown in Figure 1.5.

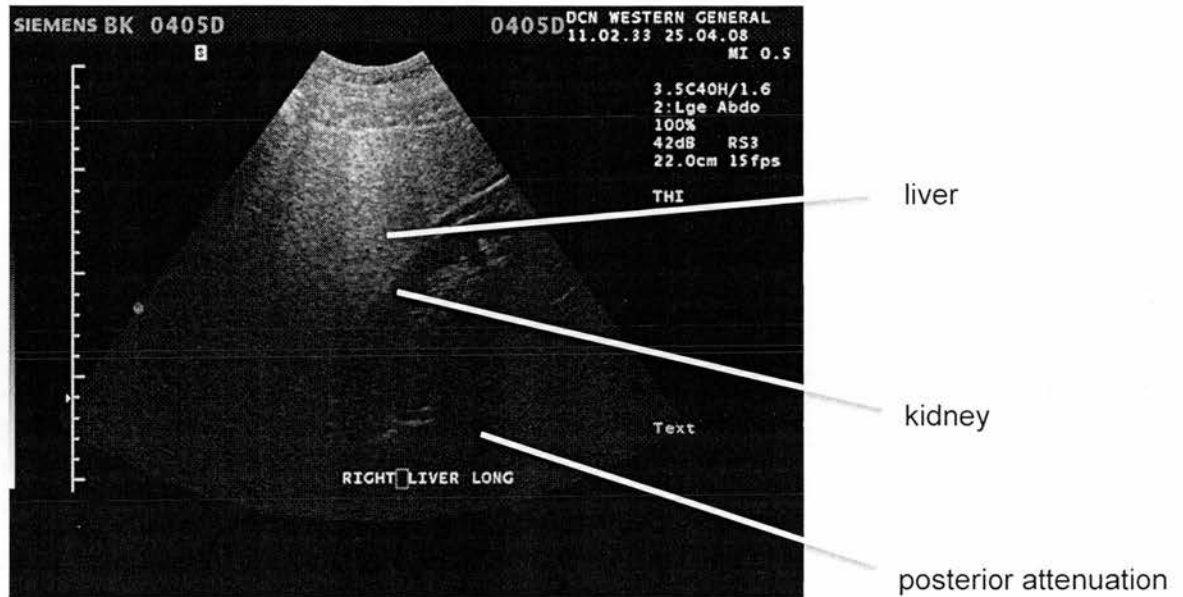
Figure 1.5

Ultrasound images of (a) a normal liver and (b) a liver with significant steatosis. In (a) the liver parenchyma and right kidney are iso-echogenic. In (b) the liver parenchyma is echogenic compared to right kidney, and there is evidence of posterior attenuation such that the diaphragm is poorly seen.

(a)



(b)



The diagnostic accuracy of ultrasound in the diagnosis of hepatic steatosis has been the subject of a number of studies. In a large meta-analysis sensitivity of ultrasound in diagnosis of any degree of hepatic steatosis was 73.3% and corresponding specificity was 84.4%(78). However, diagnostic accuracy has varied between studies, with sensitivity reported as 53.3 – 100% and specificity as 77 – 100%(75;76;80;82;84;86). There are several plausible reasons for the differing results. Firstly, studies varied considerably in the population examined, with examples ranging from participants suspected of having chronic liver disease to those at low risk such as prospective liver transplant donors. Thus the control participants who were free of steatosis comprised people with truly normal livers in some studies(75), and patients with another form of liver pathology in others(82;84;86). Secondly, ultrasound has been shown to be less sensitive in the diagnosis of steatosis in patients with significant obesity(87), but BMI was poorly documented in the studies performed. Thirdly, there was no unifying grading system between reports for the definition of ultrasound-detected steatosis. Furthermore, studies have been disparate in terms of the year in which they were performed (with a gap of twenty years separating the oldest and youngest publications included above) and therefore presumably in terms of the scanning technology utilised. This has not, however, always translated into better diagnostic accuracy in recent years, with sensitivity towards the lower end of the quoted range in the latest studies(75). A possible explanation is that the definition

of steatosis on liver biopsy has been inconsistent between studies, and indeed that in some (often earlier) reports further definition is not given. One recent prospective study of 161 prospective liver donors illustrates the potential effect of differing steatosis definitions within the biopsy comparator on the utility of ultrasound: when a cut-off of > 5% was used, sensitivity and specificity of ultrasound were 61.7% and 81.2%; corresponding figures for a cut-off of 30% were 81.8% and 98.0% respectively. This suggests that ultrasound is more sensitive in the detection of more severe hepatic steatosis, a finding mirrored in a meta-analysis in which the sensitivity of ultrasound rose from 73.3% for detecting even minimal steatosis to 85.7% for detection of steatosis > 25-33%(78).

Although a degree of subjectivity is recognised when diagnosing hepatic steatosis by ultrasound examination, few studies have formally examined intra- and inter-observer variability in making this diagnosis. One study of 25 patients with biopsy-proven NAFLD (less than a quarter of whom had diabetes) reported that the inter-observer correlation between two radiologists in determining severity of steatosis was 0.40 (representing fair agreement), and the intra-observer correlation was 0.63 (suggesting more substantial agreement)(80). A different study - of 168 patients - who had undergone abdominal ultrasonography for a variety of abdominal disorders, demonstrated inter-observer agreement between three radiologists on the presence and severity of hepatic steatosis, of 0.40-0.51 and intra-observer agreement of 0.58 (moderate agreement)(88). In both studies, the assessment of the severity of steatosis was based on the presence and severity of increased echogenicity of the hepatic parenchyma and attenuation of the ultrasound beam.

#### **1.4.4 Liver enzymes**

Screening for liver disease clinically usually involves performing (inappropriately named) “liver function tests” (LFTs), including a combination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AlkP) and gamma glutamyltransferase (GGT). These enzymes are released from damaged hepatocytes into the circulation and are therefore used as indicators of liver injury. ALT and, to a lesser extent, GGT have been used as surrogate markers of NAFLD(89;90). Laboratory reference ranges for liver enzymes rely upon historical data generated from general populations that may have included people with covert liver disease (particularly undiagnosed NAFLD). Thus, the upper part of the traditional ALT reference range may reflect values that are more typical of liver disorder than of normality. As a result, new reference ranges have been derived that are

based on data from an Italian population at low risk for liver disease (no medication use and normal BMI, lipid profile and glucose); the upper limit of normal (95<sup>th</sup> percentile) of ALT in this group was 30 units/l in men and 19 units/l in women(91). These more stringent cut-offs have not, however, been widely adopted.

The diagnostic utility of LFTs in detecting NAFLD-related hepatic steatosis has been examined in a number of studies. On the one hand, ALT levels have been shown to be related to the presence and severity of hepatic steatosis in the context of NAFLD. There is a documented stepwise increase in ALT through groups with increasing severity of ultrasound-diagnosed steatosis(92), and the prevalence of elevated ALT has been shown to be higher in people with hepatic steatosis(63;93). On the other hand, within population-based studies, the significant majority of people with hepatic steatosis have been shown to have an ALT level within the normal range(63) and mean levels have also been normal(92). There are few analyses that have formally examined the diagnostic accuracy of ALT. In one study, using the laboratory cut-off of 39 units/l to denote an abnormal ALT level, and comparing with ultrasound findings of steatosis, the sensitivity and specificity were found to be 8.2% and 98% respectively(93). The use of a lower ALT cut-off of 19.5 units/l, derived from a receiver operating characteristic (ROC) curve, gave sensitivity of 72% and specificity of 60%. In another study, in which the estimated prevalence of hepatic steatosis was estimated to be around 60%, the PPV of a high ALT level in predicting the presence of steatosis on ultrasonography was calculated to be 78%(94).

Several studies have examined the ability of LFTs, particularly ALT, to detect more advanced liver disease. ALT has been shown to be independently predictive of NASH(95) and, in a number of studies, levels have been found to be significantly higher in patients with hepatic fibrosis compared to those with less severe liver disease(96-98). However, such rises appear to be of statistical rather than clinical significance with mean values often remaining within the normal range. Certainly, the full spectrum of NAFLD has been described in people with normal ALT levels. Two studies have compared histological findings in patients with biopsy-proven NAFLD, subdivided on the basis of normal versus high ALT levels, and found no difference in the prevalence of severe fibrosis between the groups(64;96). In one of these, 6 of 51 participants with a normal ALT level had evidence of cirrhosis(64). Furthermore, in a longitudinal study comparing serial liver biopsies, mean ALT improved over time and this was associated with improvement in inflammatory grade but was independent of fibrosis progression(99).

It is noteworthy that the patients with normal ALT levels in the above studies had been referred for biopsy on the basis of clinical or radiological evidence of liver disease, and therefore represent a biased sample that may not be typical of the large proportion of NAFLD that is subclinical. This limitation is only partly addressed by a further study of routine liver biopsies from obese women undergoing bariatric surgery, over 70% of whom had a normal ALT(97). Using their laboratory cut-off of 30 units/l to denote a normal ALT, 57.9% of patients with NASH, and 31.9% of those with significant (bridging fibrosis or cirrhosis) fibrosis had a normal level. Using the same cut-off, sensitivity of ALT for diagnosis of NASH or portal fibrosis (vs. simple steatosis alone) was 32%, specificity 81%, PPV 75% and NPV 40%: corresponding figures for a cut-off of 19 units/l, as has been proposed as a more realistic upper limit of normal(91), were 65%, 40%, 66% and 40%, and this lower cut-off did not therefore appear to add diagnostic value. Again, this was a highly selected population, with a low prevalence of normal liver biopsies, and results may not be generalizable to the general population of patients with NAFLD.

In summary, it has been repeatedly demonstrated that the full spectrum of histological abnormalities is possible in people with normal ALT levels. Although there is an association between ALT levels and severity of steatosis and inflammation, this is not adequate to be able to predict individual biopsy results, and in particular stage of fibrosis appears to be relatively independent of ALT levels.

## **1.5 Detection of hepatic fibrosis and cirrhosis – non-invasive markers**

The poor performance of liver enzymes in detecting advanced liver disease has been described in Chapter 1.4. Although these remain the first line screening tool for hepatic pathology, other methods for identifying these patients have been explored.

### **1.5.1 Hyaluronic acid – a biochemical marker of fibrosis**

Extensive research has gone into the pursuit of specialised biochemical markers that reliably distinguish patients with NAFLD-related fibrosis from those with less severe forms of the condition. Hyaluronic acid (HA) is a component of the extracellular matrix, whose production is increased, and degradation by hepatic sinusoidal endothelial cells decreased, in hepatic fibrosis. Several reports have confirmed significantly higher levels of HA in patients with severe fibrosis due to a variety of causes of liver disease(100-102).

Studies have examined the use of HA as an isolated measurement in predicting severe fibrosis in NAFLD, and cut-off values of 42-50 ng/ml have been shown to give optimal sensitivity and specificity in this regard(102-105). One study of 79 patients with histologically-confirmed NAFLD, 25% of whom had severe (stage 3 – 4 on Brunt's staging system) fibrosis, a cut-off of HA of 46.1 ng/ml was associated with a sensitivity of 85% and specificity of 80% in diagnosis of severe fibrosis(102). PPV and NPV were calculated, on the basis of a 20% fibrosis rate, to be 51% and 95.5% respectively. Similar, if variable, results were obtained from the other studies with quoted sensitivity 65 – 100%, specificity 80 – 90%, PPV 51 – 92% and NPV 61 – 96%. The variation in results may be at least in part due to the prevalence of fibrosis within the cohorts studied, with the highest PPV and lowest NPV originating from the study with the highest prevalence of severe fibrosis(105). Of note, one further study has found excellent sensitivity, specificity, PPV and NPV each of over 90% using a much higher cut-off of HA (149 ng/ml) to detect any level of fibrosis(106). The reason for the discrepancy between this and the other studies is unclear, but may relate to differences in population composition or assay methodology, the latter possibly being more likely.

Higher levels of HA may be associated with increasing severity of liver disease and increased specificity in diagnosing fibrosis and cirrhosis. One study, performed in a Scottish population of patients with a variety of underlying diagnoses suggested that a level > 100 ng/ml had a 78% specificity and 83% sensitivity for predicting cirrhosis, with specificity being raised to 96% if a cut-off of 300 ng/ml was used(107).

### **1.5.2 Alternative biochemical markers of fibrosis and NASH**

In addition to the studies outlined above, the use of HA has been well documented within the European Liver Fibrosis (ELF) panel that combines three markers of matrix turnover (including tissue inhibitor of matrix metalloproteinase 1 (TIMP 1) and aminoterminal peptide of pro-collagen III (P3NP)). Together these markers have a high accuracy for severe liver fibrosis in the populations studied including patients with NAFLD(108;109). In a multicentre cross-sectional study of 1021 participants, 61 of whom had “fatty liver” and 19 cryptogenic cirrhosis (with the majority of the remaining diagnoses comprising chronic hepatitis C), the area under the ROC curve (AUC) for an algorithm containing these measures along with age was 0.862 for the test group and 0.804 for the validation group(109). For the subgroup with NAFLD the AUC was 0.870, and using the optimal cut-off, stage 3 and 4 fibrosis could be distinguished from more minimal fibrosis with sensitivity 96% and specificity 89%. It is unclear from this study what the added value of TIMP 1 and P3NP was, over and above HA. In a follow-on study undertaken in a population of people with NAFLD, of whom 23% had stage 3-4 fibrosis, the use of the ELF panel (without an age parameter) was associated with a sensitivity of 80%, specificity 90%, PPV 71% and NPV 94% in the detection of this level of fibrosis(108). It is worthwhile emphasising that these PPV and NPV are based on prevalence of fibrosis in the population studied and will be inaccurate in a population in which rates of fibrosis differ significantly. This is particularly relevant as the scores were developed in patients in whom liver enzymes were elevated and there was clinical justification for liver biopsy; in the general population PPV will be significantly lower and NPV significantly higher than in a tertiary referral centre.

Other panels of biochemical markers (including extracellular matrix molecules and enzymes) that were originally developed to stage hepatitis C have been studied in NAFLD(110). AUC for each of the following batteries of biomarkers was between 0.7 and 0.8: the Fibrotest panel contains age, sex, bilirubin, GGT,  $\alpha$ 2-macroglobulin ( $\alpha$ 2M, a protease inhibitor),

haptoglobin and apolipoprotein A1 (AUC 0.78); the Hepascore contains age, sex, bilirubin, GGT,  $\alpha$ 2M and HA (AUC 0.75); the FibrospectII panel contains  $\alpha$ 2M, HA and TIMP1 (AUC 0.70; and, finally, Fib-4 contains age, AST, ALT and platelets (AUC 0.80).

Additional to biomarkers of fibrosis, an accurate non-invasive diagnostic test for NASH would clearly be valuable in the earlier identification of people that are at increased risk of disease progression to fibrosis and cirrhosis. Cytokeratin-18 fragments (a product of hepatocyte apoptosis) hold promise in this regard. Their level has been shown to be significantly higher in patients with definite NASH compared to simple steatosis alone or healthy controls, with an AUC of 0.93(111). The exclusion of patients with “borderline NASH” from analysis represents a limitation of this study, and indeed the AUC was lower (although still indicative of good diagnostic ability) in a subsequent validation study(112).

### **1.5.3 Imaging studies in the diagnosis of hepatic fibrosis and cirrhosis**

#### *Novel techniques in the detection of hepatic fibrosis*

Transient elastography, a method of measuring tissue elasticity based on ultrasound technology, has been used in the non-invasive detection of hepatic fibrosis. Initially developed in the field of hepatitis C, it has been shown to have good accuracy in detecting severe fibrosis (stage 3 – 4) in that patient group, with an AUC of 0.90(113). Its utility is reduced in obese patients and one study showed a BMI of over 28 kg/m<sup>2</sup> to be the only independent risk factor for failure of the procedure, with an odds ratio for procedural failure of 10(114). A recent meta-analysis has suggested that an unreliable reading or failure rate might be expected in up to 25% of the obese population, even using technology such as the extra-large probe(115). Nonetheless, AUC for diagnosis of stage 3 – 4 fibrosis in the NAFLD population was high at between 0.9 and 1.0 in each of five studies examined.

In the last six years magnetic resonance elastography, a further technique by which liver stiffness can be measured, has shown promise in the detection of hepatic fibrosis. An AUC as high as 0.997 in the detection of severe fibrosis has been quoted and there are indications that it may also have a role in the detection of NASH(116;117). Its use at present, however,



is confined to research and it seems likely that cost will remain a barrier to its widespread adoption for the foreseeable future.

#### *Ultrasound imaging in detection of cirrhosis*

The diagnostic accuracy of ultrasound, as compared with histological findings, in detecting cirrhosis has been investigated in a number of previous studies. Results have been variable, with sensitivity ranging from 55% to 100% and specificity 88 to 100%(82;84;118). Diagnostic accuracy has been estimated to be 84% in one study, with PPV 79% and NPV 86%(119). The variation is likely to reflect improvements in scanning technology over time, with the poorer results being from earlier studies(118;120). When considering the discrepancies in specificity, it is important to consider the possibility of sampling error with liver biopsy – i.e. these may not be false positives, but rather true positives that have been missed by biopsy.

#### **1.5.4 Indirect measures of portal hypertension**

Both splenomegaly and thrombocytopenia are established consequences of cirrhosis. Splenomegaly arises as a result of portal hypertension. The aetiology of thrombocytopenia is multifactorial with contributors including pooling of platelets within an enlarged spleen and decreased activity of the haematopoietic growth factor thrombopoetin(121). Using a cut-off of  $150 \times 10^3/\text{mm}$ , platelet count has been shown to have a sensitivity of 53 – 68%, specificity 76 – 78%, PPV 52 – 54% and NPV 77 – 87% in detecting cirrhosis in a population of patients with chronic viral hepatitis(122). Ability to generalise these results to other forms of chronic liver disease is limited by the ability of viral hepatitis to cause thrombocytopenia by different mechanisms including direct effects on the bone marrow. The platelet count/spleen diameter ratio (PSR) is a parameter that has been established to link thrombocytopenia to spleen size, thus taking into consideration the decrease in platelet count that depends on hypersplenism rather than other factors associated with chronic liver disease(123). Its use has been developed in people with cirrhosis of varying aetiologies as a surrogate marker of portal hypertension and a means of predicting presence of oesophageal varices. Taking into account the original population of patients with documented cirrhosis and a subsequent validation study, the optimal cut-off gave sensitivity 91.5 - 100%, specificity 67 - 93%, PPV 77 - 96% and NPV 87 - 100% in the detection of varices and diagnostic accuracy was

superior to either variable alone(123;124). When patients with *compensated* cirrhosis were considered, NPV was 100% and PPV was 74%.

### Epidemiology of NAFLD

#### 2.1 Prevalence of NAFLD

##### 2.1.1 Prevalence of NAFLD

The prevalence of NAFLD has been determined in a number of studies which have been diverse in terms of populations examined and mechanism of diagnosis. These are summarised in Table 2.1.

Table 2.1

Summary of studies that have examined the prevalence of NAFLD in non-diabetic populations, I

Study	Country	Population (n)	Prevalence of diabetes	Method of diagnosis
Hilden et al, 1977(125)	Denmark	Victims of fatal road traffic accidents (503)	ND	Biopsy
Garcia-Monzon et al, 2000(126)	Spain	Obese patients undergoing bariatric surgery (46)	13%	Biopsy
Clark et al, 2003(89)	USA	General population (15676)	ND	LFTs (abnormal ALT or AST)
Abrams et al, 2004(127)	USA	Obese patients undergoing bariatric surgery (195)	35.9%	Biopsy
Browning et al, 2004(63)	USA	General population, weighted to include 50% black and 50% non-black participants (2287)	9% of white participants, 20% non-white participants	MRS

Kagansky et al, 2004(128)	Israel	People over the age of 80 years hospitalised in a rehabilitation department (91)	24%	USS
Papadia et al, 2004(129)	Italy	Obese patients undergoing bariatric surgery (2645)	ND	Biopsy
Bedogni et al, 2005(20)	Italy	General population, with participants with an elevated ALT or GGT (311) paired with participants with normal ALT and GGT (287; total 598)	ND	USS
Fan et al, 2005(130)	China	General population (3175)	16.1%	USS
Hamaguchi et al, 2005(19)	Japan	People screened as part of a health check program (4401)	ND	USS
Harnois et al, 2005(131)	France	Morbidly obese patients undergoing bariatric surgery (92)	11.9%	Biopsy

Lima et al, 2005(132)	Brazil	Morbidly obese patients undergoing bariatric surgery (112)	27.7%	Biopsy
Pendino et al, 2005(133)	Italy	General population (1645)	ND	LFTs (abnormal ALT, AST or GGT)
Jimba et al, 2005(134)	Japan	People screened as part of a health check program (1950)	5.7%	USS
Wolf et al, 2005(135)	Germany	Morbidly obese patients undergoing bariatric surgery (179)	15.6%	Biopsy
Chen et al, 2006(136)	Taiwan	General population (3245)	ND	USS
Halon et al, 2006(137)	Poland	Cadaveric liver donors (70)	70	Biopsy
Tran et al, 2006(138)	USA	Living potential liver donors (70)	ND	Biopsy

Zelber-Sagi et al, 2006(93)	Israel	General population sampled from national population registry (326)	ND	USS
Amarapurkar et al, 2007(139)	India	General population (1168)	4%	USS
Yamamoto et al, 2007(140)	Japan	Living liver donors (263)	ND	Biopsy
Williams et al, 2011(95)	USA	General population (328)	16.5%	USS (biopsy if hepatic steatosis on USS)

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; LFT, liver function test; MI, metabolic index; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ND, not done

In the above studies, the estimated prevalence of NAFLD in the general population ranged between 3.1% and 46.2%. The lowest estimates originated from two large population studies which used LFTs as surrogate markers of hepatic steatosis(89;133), and found 3.1% and 5.5% respectively to have evidence of biochemical abnormalities without a secondary cause. As has been discussed above, LFTs are poorly sensitive markers of liver injury and these figures are almost certainly underestimates of the true prevalence of NAFLD.

The majority of studies used imaging to detect hepatic steatosis and clinical and biochemical assessment to exclude secondary causes for this. Reported prevalence of NAFLD in these studies ranged between 11.5% and 46.2%. The lowest prevalence in this group was in a large population-based study in Taiwan, with exclusion of alcohol excess, hepatotoxic medication and chronic viral hepatitis from the diagnosis of NAFLD after ultrasound scanning (USS) had been performed(136). Asian countries displayed prevalences of NAFLD of under 20%, generally lower than in “Western” countries. Highest prevalences were seen in a small population of hospitalised octogenarian patients in Israel(128) and in a moderately large general population in the USA(95). In both of these studies people were excluded prior to study commencement if a secondary cause of liver disease was detected.

One further large population study merits further discussion. Browning et al examined the prevalence of hepatic steatosis in a large American population of over 2000 people taking part in the Dallas Heart Study(63). MRS, the non-invasive gold standard method of quantifying hepatic fat, was used and detailed analysis was performed to determine the normal range for hepatic fat as outlined above. The prevalence of hepatic steatosis reported, 31%, is therefore possibly the most accurate that has been reported at a population level. However, the study population was biased to include 50% black and 50% nonblack subjects and is therefore not fully representative of the Dallas population as a whole (after statistical correction for this, the prevalence of hepatic steatosis in Dallas was calculated to be 34%). In addition, due to the weight limit of the scanner, the most obese participants (n = 58) were excluded and it is therefore likely that the rate of steatosis noted is an underestimate. Formal exclusion of secondary causes for hepatic steatosis was not performed, but it was commented that the prevalence of hepatic steatosis was not different in participants who abstained from alcohol (32%) compared to those with modest alcohol intake (< 30 g/day men, < 20 g/day



women; 30%). Alcohol intake above modest levels was seen in a tiny proportion of the overall study population (< 1%).

Histological studies within the general population have included one autopsy study, one study of dead potential liver donors and two studies of living potential liver donors. The autopsy study(125) comprised people who had died in a car accident, and the prevalence of hepatic steatosis (23.9%) may therefore be fairly representative of the general population at that time. Secondary causes were not excluded, but of note, there was no correlation between the presence of steatosis with any of information on drinking habits, blood alcohol concentration at the time of the accident or circumstances of the accident, and the prevalence of “chronic alcoholism” in this sample was in the order of 2%. It should be noted that this is now an historic study and lifestyle changes since that time may have rendered this figure less applicable to today’s population. In the reports of potential liver donors, studies were often small (137;138). Furthermore, there was no systematic attempt to describe exclusions for secondary causes for liver disease, but one wonders if this process was inherent in the selection of potential donors (particularly liver donors) for biopsy.

There are a number of general points to be made about the limitations inherent in many of the studies examined. Incomplete exclusion of secondary causes of hepatic steatosis was common, and often only high alcohol intake or high alcohol intake plus chronic viral hepatitis were sought. Studies varied in the timing of such exclusions (with some studies excluding these patients altogether(19;93;131;139), and others excluding them after screening from the diagnosis of NAFLD(20;89;127;133)), and this affects the overall quoted prevalence of NAFLD. Finally, a relatively small number of countries (Italy, USA and Japan in particular) have contributed a large proportion of the data available, and it is unclear how widely the results attained can be generalised to other countries. This is particularly important as rates of excess alcohol intake and chronic viral hepatitis vary significantly between countries thus affecting exclusions from, and quoted prevalence of, NAFLD. It is also clear that the prevalence will depend considerably on rates of obesity which differ between and within countries.

### **2.1.2 Prevalence of NAFLD in the obese population**

A number of studies have examined the prevalence of NAFLD in morbidly obese populations, often by means of liver biopsy at the time of bariatric surgery. Results have been variable but prevalence of NAFLD has been reported to be over 90% in high risk populations in USA, France and Spain(126;127;131). The largest study in this field(129), of over 2600 patients, in contrast, reported a much lower rate of hepatic steatosis (26%; secondary causes were not excluded), but had used a higher cut-off of hepatic fat content to define severe hepatic steatosis (> 70% hepatocytes affected). Results are therefore not directly comparable with other studies with a similar scope, and should be regarded as prevalence of very severe steatosis. One further drawback of this study was its retrospective nature, and specifically that the pathology slides were not assessed by a blinded member of the research team according to established histological scoring systems, and rather relied on historical pathology reports.

Common procedure prior to bariatric surgery is the commencement of a hypocaloric diet, but this was not commented upon in the papers quoted above. If participants were on a pre-surgery diet then it is possible that this influenced the results observed: weight loss has been associated with a reduction in hepatic steatosis(141) and may account in part for why the prevalence of hepatic steatosis in this very high risk group was not 100%; conversely, rapid severe weight loss itself has been shown to be a risk factor for NAFLD(10).

### **2.1.3 Incidence of NAFLD**

Whilst prevalence of NAFLD has been extensively studied, its incidence has been less well characterised. One study, performed in a Japanese government office population from which people with an alcohol intake > 140 g/week, hepatitis B or C or prevalent liver disease were excluded, found a cumulative incidence of ALT or AST abnormalities of 14.7% over 60 months in the 20 – 39 year age group and 8.1% in the 40 – 69 year age group(142). The inherent limitations of the use of hypertransaminasaemia as a marker of NAFLD pertain to this study. A more robust study examined 4401 Japanese participants (mean age 48 years, mean BMI 22.6 kg/m<sup>2</sup>) who were enrolled in a medical check-up program, and in whom liver disease at baseline and secondary causes of liver disease had been excluded(19). The determined incidence of ultrasound-diagnosed NAFLD over a mean time period of 414 days

was 10% in this population. It is, however, unclear how generalizable the results would be to a non-Japanese population, and there is a possible selection bias inherent in using potentially health-conscious people who have signed up for a health check.

## **2.2 Clinical and biochemical correlates of NAFLD**

### **2.2.1 Insulin resistance and features of the metabolic syndrome**

The now-irrefutable association between NAFLD and hepatic insulin resistance has been the subject of many studies. Studies that have examined this directly and in depth have used labelled glucose and a hyperinsulinaemic euglycaemic clamp to examine insulin-mediated suppression of glucose production (from the liver), thus separating the effects of hepatic insulin resistance from insulin resistance elsewhere. In one such report, comparing 12 non-obese, non-diabetic patients with NAFLD with 6 control subjects, the rate of glucose production for any given concentration of insulin was higher in subjects with NAFLD, although this did not reach statistical significance(29). In another similar study, insulin-mediated suppression of hepatic glucose production was significantly reduced in the NAFLD group vs controls(6). These reports have substantiated conjecture that there is a causal link between NAFLD and insulin resistance as postulated by previous studies that have shown a higher level of insulin resistance in NAFLD compared to controls, independent of BMI(143;144). At a cellular level, it has been shown that triglyceride accumulation within hepatocytes interferes with tyrosine phosphorylation of IRS-1 and -2 to cause insulin resistance(145). Insulin resistance at the level of the liver exacerbates whole-body insulin resistance and thus contributes to the pathogenesis of Type 2 diabetes(26); factors associated with the progression of NAFLD, particularly inflammatory cytokines as outlined in Chapter 1.3, may also promote this sequence of events.

Several studies have examined the relationship of NAFLD with clinical features that have tended to cluster with insulin resistance, together known as the metabolic syndrome. Such is the strength of the associations that NAFLD has been proposed as the hepatic component of this syndrome(6). There are several definitions of the metabolic syndrome, but all include the components of central obesity, hypertension, dyslipidaemia and glucose intolerance(26). As reported above, the prevalence of NAFLD in subgroups of people with components of the metabolic syndrome such as obesity is higher than in the general population. Both cross-sectional and (to a lesser extent) longitudinal studies have examined the relationship of NAFLD with these individual elements, and with the metabolic syndrome as a whole, in more detail. These are outlined below, and the relationship of NAFLD with Type 2 diabetes is dealt with in depth in later chapters.

## *Obesity*

Obesity has been consistently associated with the presence of hepatic steatosis and NAFLD(19;20;63;94;95;146). A number of studies have examined the prevalence of NAFLD, or surrogate markers of the same, in people grouped according to BMI, as a marker of generalised obesity. In a large study population in which alcohol excess and chronic viral hepatitis as secondary causes of steatosis had been excluded, prevalence of a raised ALT level increased in a stepwise fashion through four groups subdivided according to BMI (< 25 kg/m<sup>2</sup>, 1.0%; 25 – 30 kg/m<sup>2</sup>, 3.3%; 30 – 35 kg/m<sup>2</sup>, 5.5%; > 40 kg/m<sup>2</sup>, 6.6%)(146). Another study which examined prevalence of NAFLD as defined using ultrasound and exclusion of people on the basis of alcohol excess and viral hepatitis found a prevalence of 16.4% in people with a BMI < 25 kg/m<sup>2</sup> compared with a prevalence of 75.8% in those with BMI > 30 kg/m<sup>2</sup>, a 4.6-fold difference(94). One of the first papers to examine the relationship between obesity and hepatic steatosis on autopsy specimens from patients without a known secondary cause for fatty liver showed an increase in prevalence of steatosis and in the proportion of more significant steatosis (>25% of hepatocytes involved) in a stepwise fashion through increasing grades of obesity(147).

Conversely, other studies have compared BMI between people with a normal liver and those with NAFLD, and have found significantly higher BMI levels and rates of obesity in participants with presumed NAFLD (20;63;95). In a study of over 2000 members of the general population of the USA, the prevalence of obesity of 67% in those with hepatic steatosis, compared with 33% in the subgroup without steatosis(63). Another large study carried out in Japan showed a similar trend, with a prevalence of high BMI (>25 kg/m<sup>2</sup>) of 54% in the group with NAFLD versus 15% in the group with a normal liver(19). Biopsy studies have consistently demonstrated a positive correlation of hepatic steatosis (quantified according to percentage of hepatocytes affected) and BMI(129;148).

The link between abdominal/visceral adiposity is particularly strong and has been investigated in studies that have used both indirect and direct measures of abdominal obesity. In parallel with BMI, both WC and WHR have been shown to be associated with NAFLD(20;146). In one multivariate analysis, looking at predictors of a raised ALT level, the association of BMI with NAFLD was ameliorated by the addition of WHR to the model, and WHR was the variable most consistently associated with NAFLD even after addition of

insulin concentration to the model(146). Similar conclusions were drawn from a second study in which WC, but not BMI, was an independent predictor of the presence of NAFLD as diagnosed using ultrasound(93). Other studies have used cross-sectional imaging to directly measure visceral adiposity. One such study showed that the relationship between BMI (and indeed WC) and NAFLD, was eliminated after adjustment for visceral adiposity quantified on computerised tomography (CT) imaging, whereas abdominal subcutaneous fat values did not modify the association - suggesting that fat deposition around the intra-abdominal organs is the more important determinant of NAFLD(149). Of note, this study used a definition of NAFLD that required both hepatic steatosis on CT scan and an elevated ALT level and it is not possible to clarify the relative importance of hepatic steatosis per se, as opposed to the inflammatory process that may possibly be inferred by high ALT levels, on this relationship

### *Triglycerides*

Triglyceride concentrations have been associated with NAFLD independently of obesity in some studies(20;93;130;134) but not in others(146). Triglyceride levels and the presence of abdominal obesity are intrinsically interlinked, as described in Chapter 1.3, and in multivariate models the effect of one can be at least partly offset by the other. The specific association between hepatic steatosis and triglyceride levels, rather than between steatosis and hyperlipidaemia generally, has been demonstrated in a study of patients referred to a lipid clinic. The prevalence of ultrasound-diagnosed fatty liver was 72.4% in the sub-group with hypertriglyceridaemia, compared to 32.7% in the sub-group with hypercholesterolaemia and normal triglyceride levels. Triglyceride was a predictor for hepatic steatosis independently of obesity (BMI > 28) and the presence of diabetes. Limitations were the failure to exclude participants with excessive alcohol intake which can be associated with both hypertriglyceridaemia (although alcohol was not significantly associated with hepatic steatosis in this study) and the lack of a measure of abdominal adiposity.

### *Metabolic Syndrome*

The prevalence of NAFLD has been shown to be significantly higher in people with the metabolic syndrome than in those for whom the criteria are not met. In one study, the prevalence of an elevated ALT level was 6.3% in people with the metabolic syndrome and

2.0% in those without ( $p = 0.005$ )(146). Another study examined the prevalence of features of the metabolic syndrome in 304 patients with NAFLD, and found that a diagnosis of metabolic syndrome could be made in 36% of cases (this rose to 67% of patients in the obese category)(150). In a study of the prevalence and incidence of NAFLD in a large Japanese population, NAFLD at baseline was associated with a higher prevalence of each of the five components of the metabolic syndrome, and a significantly ( $5 - 9 \times$ ) higher prevalence of the metabolic syndrome overall when compared to those with a normal liver on ultrasound(19). Furthermore, in participants without NAFLD at baseline, the presence of the metabolic syndrome was associated with an adjusted odds ratio of developing NAFLD of 4.00 (2.63 – 6.08) in men and 11.20 (4.85 – 25.87) in women, independent of weight gain.

## **2.2.2 Demographics**

### *Age*

Results from studies that have analysed the association between age and NAFLD have not been uniform. Some have found an association of younger age with NAFLD both on univariate analysis and after adjusting for confounders(20;146). Pathophysiologically it is difficult to explain age as a protective factor, and it is instead more likely that results are skewed due to morbidity and mortality secondary to NAFLD or other medical conditions associated with NAFLD or obesity such as cardiovascular disease or cancer. Thus survival bias may be playing a role in the association. Another possible explanation is that more severe fibrotic liver disease is being missed in these older individuals, as steatosis regresses with increasing fibrosis. Other studies within the general population have found no association or a positive relationship (63;95) of age with NAFLD.

Of interest, the one study that has specifically looked at an older population (octogenarians undergoing rehabilitation in hospital) found a high prevalence of ultrasound-diagnosed NAFLD overall (46%, after appropriate exclusions), but did not find any additional effect of age within the cohort(128). When this group was compared with another group with mean age 54 years, also diagnosed with NAFLD, the prevalence of obesity (as measured by BMI), glucose intolerance, hypertriglyceridaemia and metabolic syndrome was significantly less in the older cohort. The prevalence of a raised WC, however, was not different. One might conjecture that age is associated with a weakened association between the metabolic

syndrome and NAFLD, perhaps due to a different, age-related, mechanism for hepatic steatosis. The conclusions that can be drawn are, however, weakened by the study design, whereby the older and younger groups with NAFLD were not comparable in terms of selection (with the older group picked up by population screening, and the younger group diagnosed in the process of investigation for abnormal LFTs).

### *Gender*

In contrast to early, biased data that suggested that NAFLD was a disease more prevalent in women, male gender has been shown to be associated with the condition in a number of more representative studies(63;93;95;146). One possible reason for this is the higher rate of abdominal/visceral obesity in men (compared with a higher rate of subcutaneous adiposity in women), and in one of these studies the association was lost after adjustment for WC or WHR(146). In other studies, however, correction for obesity and insulin resistance differences between the sexes has not abolished the relationship of male gender with NAFLD(63;93;95). One study which examined the effect of gender on the presence of hepatic steatosis as quantified using MRS found an almost two-fold higher prevalence of hepatic steatosis in white men compared to white women(63). This significantly higher prevalence remained even when a non-obese (BMI < 30) and relatively more insulin sensitive (HOMA IR < 4.04) subgroup was examined. Although their obesity measure did not take into consideration the differential effects of a greater degree of visceral adiposity in men, the results within the insulin-sensitive subgroup suggest that another factor is involved.

One possible explanation is different rates of alcohol consumption between the genders. In this study men who reported a moderate intake of alcohol (< 30 g/day) had a higher prevalence of hepatic steatosis than their non-drinking male counterparts: the converse was true in women (alcohol < 20 g/day). Although the hepatic metabolism of ethanol is known to be sexually dimorphic, it is unclear why the prevalence of hepatic steatosis was raised in men and not women with moderate alcohol intake. In this study obesity was not a confounder. It has been conjectured that ethanol intake in women may be associated with an increase in insulin sensitivity whereas in men this is less pronounced and is outweighed by hepatic triglyceride accumulation.



An alternative explanation is that sex steroid hormones play a direct role in the pathogenesis of NAFLD. This hypothesis is supported by a Chinese study which showed a higher prevalence of NAFLD in men than women under the age of 50 years, but this effect disappeared above 50 years, possibly suggesting a protective effect of oestrogen in pre-menopausal women(130). These results have been mirrored in a study within the National Health and Nutrition Examination Study (NHANES) III carried out within the USA which not only showed that the prevalence of NAFLD (diagnosed on the basis of liver enzyme elevation) was two-fold higher in post-menopausal women than in pre-menopausal women, but also that the use of hormone replacement therapy in post-menopausal women was associated with a significantly lower prevalence of NAFLD(151). Moreover, tamoxifen (an oestrogen receptor antagonist) has also been implicated in drug-induced hepatic steatosis(152). Aside from a possibly protective role of oestrogen, a deleterious role for androgens is suggested by the higher prevalence of NAFLD in women with polycystic ovarian syndrome compared with the general population(153-155).

## **2.3 Relationship of NAFLD with Type 2 diabetes**

The pathogenesis of NAFLD and Type 2 diabetes are intrinsically interlinked, and this relationship has been examined in some detail. The central role of insulin resistance in promoting the development of hepatic steatosis has been described above. In the present chapter the epidemiological evidence linking NAFLD and Type 2 diabetes will be explored in three sections: (1) cross-sectional studies that have assessed the association of NAFLD with Type 2 diabetes within the general population; (2) longitudinal studies that have indicated a role for NAFLD in the development of Type 2 diabetes; and (3) studies that have examined the prevalence of NAFLD in populations of people with Type 2 diabetes. Evidence suggesting a role for diabetes in promoting increasing severity of NAFLD is considered in subsequent chapters.

### **2.3.1 Cross-sectional associations of NAFLD and Type 2 diabetes**

Several studies, many of which have been described above, have shown a higher prevalence of diabetes within groups diagnosed with NAFLD than in those with no evidence of liver disease. Two large-scale population studies within the USA showed a significant association between ALT elevation (without causative alcohol excess or viral hepatitis) and the presence of diabetes(89;146). In a further American study, ultrasound-diagnosed NAFLD (alcohol excess and hepatotoxic medications excluded) was associated with a significantly higher prevalence of history of diabetes compared to a normal liver (26.3% vs 7.9%)(95). Similarly, in the Dallas Heart Study the presence of hepatic steatosis on MRS was associated with a significantly higher prevalence of impaired fasting glucose or diabetes (18%, vs 11% in those with no steatosis; defined as a one-off plasma glucose  $\geq 6.1$  mmol/l)(63). There was also a significant positive correlation between hepatic triglyceride content and fasting plasma glucose. In a study of patients within a metabolic clinic there was a stepwise increase in the prevalence of diabetes in groups with increasing severity of NAFLD on ultrasound, albeit that the semi-quantitative grading for steatosis was unvalidated (normal liver, 10.2%; mild steatosis, 23.1%; moderate steatosis, 32.1%; severe steatosis, 40.6%)(92). This significant association remained valid after adjusting for obesity and hypertriglyceridaemia. The finding of a significant association between hyperglycaemia/diabetes and NAFLD has been replicated in a number of other reports(19;20;93;130). Differences in the exact prevalence of hyperglycaemia and diabetes within the NAFLD cohorts studied are likely a consequence of differing study populations (in terms of ethnicity and inclusion criteria), varying exclusion of

secondary causes for hepatic steatosis and inconsistent criteria for the diagnosis of diabetes (for example, the use of fasting glucose versus a formal oral glucose tolerance test versus a clinical history of diabetes).

### **2.3.2 Epidemiological evidence for a role for NAFLD in the development of type 2 diabetes**

There is a growing body of evidence that NAFLD precedes the onset of type 2 diabetes, and indeed may have a crucial role in its pathogenesis. The first studies to examine this relationship used LFTs as surrogate markers of fatty liver, but subsequent studies have used more reliable ultrasound measures.

An early prospective study of 766 middle-aged Swedish men, followed up over 13 years, showed significantly higher levels of baseline transaminases in those that developed diabetes (6.1% of the population) compared with those that did not(156). Those in the top quintile of glutamic pyruvic transaminase at baseline had a relative risk of developing diabetes of 3.9 when compared to those in the bottom quintile. The association remained significant after adjustment for BMI, WHR and blood glucose at baseline. Results from a larger study within the UK, The British Regional Heart Study, were similar(157). In 3500 middle-aged, non-diabetic men, followed up for a mean of 5 years, ALT levels at baseline were significantly higher in the 100 participants who developed diabetes (as determined using the National Health Service central registers) than in those who did not (19.3 IU/l vs. 15.3 IU/l). This association persisted after adjustments for other biometric variables including BMI and WC; the exclusion of heavy drinkers made little difference to the relationship and when entered into the multivariate analysis alcohol intake did not disturb the relationship. A further study on the role of liver dysfunction in the development of type 2 diabetes in the Pima Indian population yielded comparable results(158). Of 370 participants with normal glucose tolerance at baseline 63 developed diabetes. The relative risk of progression to diabetes of those in the ninetieth percentile (ALT 70 iu/l) compared to the tenth percentile (ALT 12 iu/l) was 2.3 (1.4 – 3.6). The predictive effect of a high ALT persisted after adjustment for percentage body fat, whole-body insulin resistance on clamp study, insulin response to a 25g intravenous glucose load and hepatic insulin sensitivity measured by hepatic glucose output during insulin infusion.

Other studies that have focused on younger(159) and more generalised populations(160) have produced similar results. Only one study had the advantage of strict formal exclusion of people with a secondary cause for liver disease from the “NAFLD” cohort(160).

The West of Scotland Coronary Prevention Study (WOSCOPS) took investigation of the relationship between ALT and development of diabetes a step further by examining not only the effect of baseline ALT on subsequent incipient diabetes, but also serial metabolic measurements leading up to diabetes diagnosis. In a cohort of almost 6000 men, from whom those with a known history of diabetes, fasting blood glucose  $\geq 7.0$  mmol/l or ALT  $\geq 70$  units/l at baseline were excluded, 139 (2.33%) were diagnosed with diabetes over a mean time of 4.9 years(161). Mean ALT at baseline was 18% higher in those who progressed to diabetes and men in the top quartile (ALT  $\geq 29$  units/l, and therefore well within the normal range) had a significantly increased risk of developing diabetes relative to those in the lowest quartile, even after adjustment for multiple physiological and metabolic variables including BMI, blood glucose, the presence of the metabolic syndrome and CRP. This study included alcohol intake in its multivariate analysis, although once again other causes of liver dysfunction were not excluded. On retrospective review of 6-monthly measurements (including ALT and fasting glucose) in 86 subjects who had developed diabetes along with 860 controls, conversion to diabetes was associated with a stepwise increase in ALT over the previous 18 months(162). Change in ALT was the variable that was most predictive of conversion to diabetes in this cohort. Results from this study were supported by those from a prospective study of 287 Japanese employees who were free of metabolic features or secondary causes of liver disease (alcohol excess and chronic viral hepatitis) at baseline, and were subsequently followed up annually(142). In this population, weight gain preceded hypertransaminasemia which in turn preceded glucose intolerance.

There are a number of limitations that are common to many of the above studies. Studies were often carried out in selected populations (e.g. men, Pima Indians) and it is unclear how generalizable results are to the general population of patients with Type 2 diabetes. Criteria for the diagnosis of diabetes differed between studies (often relying on fasting glucose measurements alone(156;161;162)) and exclusion of diabetes at baseline was often incompletely performed(156;157). Thus it is possible that undiagnosed diabetes at baseline may have influenced the metabolic variables. There was sometimes poor recording of

secondary causes of hepatic steatosis such as alcohol intake(156;158). Of note, the WOSCOPS study was set up as a randomised controlled trial of pravastatin therapy in people with moderately high cholesterol, and the analyses described above were post hoc and retrospective.

The association of GGT and development of Type 2 diabetes has also been examined and results have been rather more variable, with some studies suggesting a predictive role of GGT(90;157) and others not(158). In those studies where a significant association was detected it was often not possible to say if this was independent of ALT. Clearly, alcohol intake would be an important confounder and this was not universally estimated(158).

While the body of evidence described above implicates NAFLD in the pathophysiology of Type 2 diabetes, the evidence linking a more robust marker of hepatic steatosis (measured via imaging or biopsy) directly to progression to diabetes in the population has, until recently, been limited. Within the last decade there have been four studies that have examined this, all within Far Eastern countries. A study that compared the outcomes of 358 Chinese people with NAFLD with 788 controls found a significantly higher incidence of diabetes in the NAFLD group (20.3% vs 5.2%), but did not adjust for confounding variables(163). In a Japanese study of over 3000 male employees, in whom alcohol intake  $\geq$  20 g/day, hepatotoxic medication use and viral hepatitis had been excluded, the presence of ultrasound-diagnosed NAFLD at baseline was associated with a significantly higher incidence of diabetes over a mean follow-up period of around 4 years, with an adjusted (for age and BMI) hazard ratio of 5.5 (3.6 – 8.5)(164). Results from a Korean study were similar, but this study performed more thorough adjustment for metabolic variables including fasting glucose and triglycerides at baseline which may account for the lower, but still significant, relative risk of development of diabetes in the cohort with fatty liver (1.51 (1.04 – 2.20)(165). The only study that did not find a significant independent relationship between hepatic steatosis and incidence of diabetes observed a univariate association, but this was abolished by adjustment for age and fasting plasma glucose(166). However, this study did not exclude people with secondary causes of hepatic steatosis.

In summary, the early evidence linking abnormalities in liver enzymes to subsequent development of diabetes has been strengthened by studies that have used a more direct measure of NAFLD. These epidemiological reports are in keeping with the smaller, more detailed studies examining the association of NAFLD with insulin resistance at a cellular level, as outlined in Chapter 2.2. The above evidence raises the question as to why NAFLD is not a ubiquitous finding amongst patients with Type 2 diabetes. It is clear, however, that Type 2 diabetes is not a homogeneous condition, and it is likely that the primary site of insulin resistance varies between patients. Indeed it is implied that a certain initial sensitivity to insulin at the level of the liver is necessary to allow triglyceride accumulation and this may differ from person to person.

### **2.3.3 Prevalence of NAFLD in people with Type 2 diabetes**

With a rising incidence of Type 2 Diabetes and its incontrovertible association with NAFLD, an accurate estimate of the prevalence of NAFLD in this population is important for determining service provision, as well as contributing to an understanding of the underlying pathogenesis. Despite this, studies examining the prevalence of NAFLD in otherwise unselected populations of people with Type 2 diabetes have been infrequent.

As within the general population, initial studies in Type 2 diabetes used indirect means to examine the prevalence of liver disease. The prevalence of abnormal LFTs has been found to be moderately high in populations with type 2 diabetes (compared to studies described above which looked at the general population), with some variation between studies. In a study of 118 consecutive patients with Type 2 diabetes in a Finnish outpatient clinic, 22.9% had an ALT level above normal(167). Another study of over 9000 patients with Type 2 Diabetes the prevalence of raised ALT was 16%(168). In an even larger study from the USA the prevalence was 7.8%(169). The inherent limitations of using LFTs as a marker of liver disease have been described above, and it is clear that these figures represent an underestimation of the true prevalence. Furthermore, no attempts were made to exclude people with other causes for elevated LFTS.

A small number of studies have investigated the prevalence of ultrasound-diagnosed NAFLD in diabetic clinic populations. In an Indian study of 100 patients with type 2 diabetes, in

whom those with alcohol intake and viral hepatitis had been excluded, 49% were found to have steatosis on liver ultrasonography, and this was confirmed histologically on all who went forward for biopsy(170). A further study performed in India, of 124 consecutive patients with Type 2 diabetes in whom alcohol or hepatotoxic medication use or hepatitis B or C positivity were excluded, found a prevalence of NAFLD of 57.2%. The prevalence of hepatic steatosis on ultrasound examination in a small Saudi diabetic population, in which alcohol and hepatotoxic medication use was excluded, was similar at 55%(171). In a Brazilian cohort of 180 patients with Type 2 diabetes enrolled from a tertiary referral unit in whom alcohol use  $\geq 20$  g/day, hepatotoxic medication use and chronic viral hepatitis had been excluded, 69.4% had NAFLD diagnosed on ultrasound scan(172), and this was subsequently confirmed histologically in 94%(173).

The prevalence of NAFLD has been studied in a much larger population of Italian patients with type 2 diabetes, encompassing an entire type 2 diabetes clinic outpatient population. Of 2839 participants, 2421 (85.3%) had hepatic steatosis of whom 81.5% met the criteria for NAFLD (i.e. hepatic steatosis without evidence of excess alcohol consumption, viral hepatitis or causative medications). The prevalence of NAFLD in this population was therefore 69.5%(18).

In summary, the calculated prevalence of NAFLD in people with Type 2 diabetes has varied according to the diagnostic tools used, but is likely to be in the region of 50-70%. Aside from the Italian study described, studies have been small and undertaken in non-Caucasian populations (in which the prevalence of NAFLD may be dissimilar to that of a UK population), or have been large populations in which an indirect measure of NAFLD has been used. Those that have used imaging have been undertaken in hospital diabetes clinics, and it is therefore difficult to be sure that these are truly unselected populations. Validation of ultrasound detection of hepatic steatosis was done infrequently, and only in those patients with a positive ultrasound scan. Thus the specificity of ultrasound in these populations has been shown to be good, but sensitivity has not been demonstrated. Finally, exclusion of secondary causes for liver disease has often been incomplete.

## **2.4 Progression of NAFLD - prevalence of advanced liver disease and risk factors for development**

It is well recognised that NAFLD comprises a spectrum of liver disease, from simple steatosis to NASH and fibrosis. “Cryptogenic” cirrhosis has been shown to share many of the same risk factors as NAFLD and is now established as the most severe end of this spectrum(11;12). Less well understood is the prevalence of these lesions in the general population of patients with NAFLD, and the likelihood of progression to more advanced liver disease. These issues have been partially addressed in cross-sectional and longitudinal biopsy studies.

### **2.4.1 Prevalence of advanced liver disease**

#### *Cross-sectional studies*

Cross-sectional studies have estimated a prevalence of NASH of 22 – 72%, significant fibrosis of 10 – 33% and cirrhosis of 1 – 11% within cohorts of patients investigated for NAFLD on biopsy. Studies have been diverse in terms of populations studied but have broadly fallen into two categories: those that have examined biopsy specimens performed on clinical grounds; and those in which biopsies have been taken specifically to investigate the prevalence of NAFLD in high risk populations. Three studies in the former category examined similar patient groups. A retrospective study of 458 Italian patients diagnosed with NAFLD (on the basis of biopsy results, daily alcohol intake of < 20 g and negative liver screen, with the significant majority having abnormal LFTs) found a prevalence of NASH of 72% and prevalence of “severe” fibrosis (perisinusoidal and portal fibrosis, septal or bridging fibrosis or cirrhosis) of 33%(96). The prevalence of grade 3 (bridging) or 4 (cirrhosis) fibrosis were 9% and 5% respectively. A prospective study within the UK of consecutive patients diagnosed with NAFLD after biopsy for abnormal LFTs found that 52% had features of NASH. Bridging fibrosis and cirrhosis were present only in patients with NASH, and accounted for 5.4% and 4.3% of the study population respectively(174). In a larger prospective study of French patients undergoing liver biopsy during investigation for abnormal LFTs in the absence of a secondary cause for this, the prevalence of significant (stage 2 – 4, portal and septal fibrosis or cirrhosis) fibrosis was 27.4% and cirrhosis was 3.4%(175). Thus, even in ostensibly similar patient populations, with relatively similar prevalences of cirrhosis, there is variation in reported rates of fibrosis. This is likely to be



partly due to different staging systems used, with associated variability in the definition of severe or significant fibrosis. Inter-observer variability in histological examination may also play a role. As all biopsies were carried out according to clinical need, subtle differences between countries and centres in clinical practice may have influenced the results. Clearly an important limitation of these studies is the highly selected patient populations used, and the high prevalences of fibrosis and cirrhosis may be unrepresentative of the general population of patients with NAFLD, many of whom will lack features that may prompt biopsy. This latter point is illustrated by studies that have examined the results of histological examination of liver biopsies taken from people who were at low risk for liver disease. In a study of 100 prospective liver donors, who had passed clinical, imaging and serological evaluation, 6% met minimal criteria for steatohepatitis and no subject had fibrosis on biopsy(148). In a study of autopsy results from over 500 victims of road traffic accident, portal fibrosis was found in 2% (and this could not be guaranteed to be due to NAFLD) and cirrhosis in nobody(125).

Three studies have examined prevalence of advanced liver disease in subjects with significant obesity. In a prospective study, 103 consecutive patients (the majority of whom had normal ALT levels) underwent liver biopsy during bariatric surgery(176). Twenty-five percent had NASH, 11% had significant (bridging fibrosis or cirrhosis) fibrosis and only one participant had cirrhosis. In a population of 237 obese women undergoing bariatric surgery, 32.6% had NASH, 9.4% had “advanced” (bridging fibrosis or cirrhosis) fibrosis and 1% had cirrhosis(97). The rate of NASH and fibrosis in these populations appears less than in studies based within populations undergoing investigation for clinically or biochemically suspected liver disease. Interestingly, in a study of overweight patients (BMI > 25) *with* at least one LFT above the upper limit of normal, the prevalence of NAFLD-related septal fibrosis was higher at 30% including cirrhosis in 11%(177).

#### *Prevalence of NASH and fibrosis in a population of patients with Type 2 diabetes*

In the current setting the one study that has examined the prevalence of advanced liver disease in a population of patients with Type 2 diabetes is of particular interest(172). This study comprised participants under the age of 65 years, in which secondary causes for liver disease had been excluded (alcohol intake > 20 g/day, hepatotoxic drug use, viral hepatitis). All participants (n = 180) underwent ultrasound imaging of the liver and LFT evaluation and those with hepatic steatosis (n = 125) or abnormal aminotransferase levels alone (a further 5

participants) were invited to undergo liver biopsy. This population was therefore relatively less selected than those that have required abnormalities in LFTs or clinical evidence of significant liver disease to qualify for biopsy. Of those undergoing biopsy (92 of the 130 invited), 76 – 78% had NASH, and 34 – 60% had significant fibrosis (stage  $\geq 2$ ). Prevalence of Stage 3 fibrosis was 6 – 11%, and that of stage 4 fibrosis (cirrhosis) was 3%. The range of values reflects differences in grading/staging between two independent pathologists, and raises some concern regarding inter-observer variability in the staging of fibrosis. If one makes the (imperfect) assumption that those with normal imaging and biochemistry had a normal liver, and that those refusing biopsy were similar to those who underwent biopsy, the prevalences of NASH, stage 3 – 4 fibrosis and cirrhosis in the whole cohort are calculated to be 55 – 56%, 6.5 – 10% and 2.2% respectively.

### *Longitudinal studies*

There are a small number of studies that have looked at serial biopsy specimens to determine rate and predictors of progression of NAFLD. One study examined the results of 103 patients with NAFLD, with mean time between first and final biopsy of 3.2 years (range 0.7 – 21 years)(99). Repeat biopsies had been performed either as part of routine clinical care (n = 26) or as part of trials of clofibrate or ursodiol (neither of which were shown to affect outcome) in the context of NASH (n = 77). Although the performance of repeat biopsy as part of study procedure limits the bias inherent in re-biopsying in response to clinical change, the sampling at baseline was weighted towards those with NASH due to drug trial protocols, and therefore cannot be considered representative of the general population of patients with NAFLD - 93.2% had NASH, 34% had stage 3/4 fibrosis, of whom around half (16% of the entire study population) had cirrhosis. At follow-up, 67% of those biopsied more than two years apart had evidence of progressive fibrosis, and 24% of those biopsied more than 4 years apart had evidence of progression of two or more fibrosis stages. Nine participants (8.7% of the study cohort) progressed to cirrhosis, of whom two had no evidence of fibrosis at baseline. Of the three participants who had bland steatosis on initial biopsy, two developed NASH and one remained unchanged. Three smaller studies have examined outcome of patients with biopsy-proven NASH at baseline. In one, 4 of 11 (36%) patients who underwent a second liver biopsy showed evidence of progression of fibrosis, and one of these developed cirrhosis(178). A second retrospective study revealed similar results, with 5 of 13 (38%) patients with serial hepatic biopsies developing progression of fibrosis over a range of 1.7 – 6.1 years, two of whom developed cirrhosis(179). A third prospective study of

22 patients with NASH who agreed to undergo repeat biopsy after a median of 4.3 years as part of study protocol showed progression of fibrosis by at least one stage in 31.8%(180). No patient progressed to cirrhosis.

Only two studies had a significant proportion of patients with simple steatosis at baseline within the study population. One of these examined 129 subjects who had been diagnosed with biopsy-proven NAFLD during investigation for abnormal LFTs were invited to have follow-up investigations, including a repeat liver biopsy, after a mean time period of  $13.8 \pm 1.2$  years(98). At baseline, 36% had simple steatosis, 9% had steatosis with nonspecific inflammation, and 55% had NASH. In those who had a second biopsy fibrosis had progressed in 41%, but it was unclear what proportion of the progressors had NASH as opposed to simple steatosis at baseline. Another study exclusively examined outcomes of 40 patients with NAFLD-related steatosis alone, without inflammatory or fibrotic components and found no evidence of progression in 11 of 12 patients who underwent a second biopsy, with the remaining patient having evidence of moderate pericellular fibrosis(181). In the remaining 28 patients there was no evidence of liver-related morbidity or mortality but this conclusion relied on the flawed assumption that normal LFTs and imaging excludes significant fibrosis.

In summary, there is a variable incidence of progression of fibrosis in people with NAFLD, depending on the population and time course studied. It has been proposed that simple steatosis is a benign condition with little chance of progression compared with the more pathogenic NASH(181). Supporting evidence for this view, however, is limited and further investigation of serial biopsies over longer periods will be required before this can be fully established.

#### **2.4.2 Risk factors for progression**

Many of the studies outlined above have examined risk factors for NAFLD-related fibrosis and its progression. As expected, there is considerable overlap with risk factors for NAFLD itself. In this section I shall consider both clinical and histological features that are associated with hepatic fibrosis. The utility of LFTs in screening for advanced liver disease has been previously described in Chapter 1.4.

### *Diabetes is associated with increased severity of NAFLD*

The association of Type 2 diabetes with increased prevalence and severity of steatosis, necroinflammation and fibrosis has been examined in histological studies. Diabetes has been independently associated with increased severity of steatosis(176) and NASH(96;147). In a cross-sectional autopsy study, those with Type 2 diabetes had a 2.6-fold increase in the prevalence of steatohepatitis, and this appeared to be independent of the effect of obesity, with the combination of diabetes and obesity being associated with particularly high risk(147). In a number of independent studies each with over 100 participants with NAFLD, diabetes has been shown to be an independent risk factor for the presence of advanced fibrosis on biopsy(64;96;147;175;176;182). The odds ratio of diabetes in predicting severe fibrosis has been variously calculated to be between 1.8 and 4.4(96;176). In a study of 144 patients with NASH, the prevalence of diabetes rose from 14% in the sub-group with no fibrosis to 54% in the sub-group with stage 3-4 fibrosis(182). Showing a comparable trend, a study of 132 patients with biopsy-proven NAFLD estimated a prevalence of cirrhosis of 25% in those with diabetes, compared to 10% of the non-diabetic cohort(67). A similar relationship was seen in a longitudinal study of 103 patients who had undergone serial liver biopsies in which the presence of diabetes at baseline was associated with a greater rate of progression of fibrosis independently of baseline histology and obesity(99). This last study establishes a temporal relationship that is not possible in the cross-sectional studies.

A small number of studies have failed to demonstrate a link between the presence of diabetes and more severe liver disease(67;126;178-180), but these studies were often small and in some cases there was a trend that failed to reach statistical significance(67;126;180). No study, to my knowledge, has shown a correlation between the presence of diabetes and less severe liver disease.

Three studies have looked at severity of diabetes (as measured by glycaemic control or duration of diabetes) and severity of liver disease. In those that have examined glycaemic control using HbA1c there has been no difference between histological groups(167;173). In contrast, one study has suggested a link between duration of diabetes and severity of NAFLD. In this study of 32 patients with Type 2 diabetes and biopsy-proven NAFLD, the median duration of diabetes rose in a stepwise fashion from 9 months in a group with

steatosis alone to 60 months in a group with severe NASH, a statistically significant rise(170). This result has not been replicated(173).

One explanation for the association of diabetes with more advanced liver disease is that diabetes is merely a surrogate marker for longer duration of NAFLD. There are, however, studies that have suggested a direct effect of hyperglycaemia or hyperinsulinaemia on the fibrotic process. *In vitro* studies of rat hepatic stellate cells, the key players in the production of extracellular matrix proteins and fibrogenesis within the liver, have shown an up-regulation of connective tissue growth factor (CTGF) mRNA and protein production following incubation with high-concentration glucose- or insulin-containing solutions(183). As CTGF has been shown to stimulate fibrogenesis, the implication is that hyperglycaemia/hyperinsulinaemia may promote hepatic fibrosis via this pathway. The evidence for hyperglycaemia (but not hyperinsulinaemia) influencing progression of fibrosis was strengthened by results from a mouse model of NASH in which induction of diabetes using streptomycin was associated with an increase in expression of markers of fibrosis, and this was prevented by insulin treatment(184). In human subjects, indirect support for a role of hyperglycaemia in fibrosis progression comes from a study that demonstrated an association between an improvement in HbA1c and improvement in hepatic fibrosis in diabetic subjects undergoing serial assessment(185). The study was, however, small and non-randomised and the associative relationship between these variables may not necessarily imply causation.

#### *Other clinical risk factors for progression*

Obesity is commonly associated with more advanced liver disease(96;147;150;180) and in many studies is an independent predictor of progression, over and above the presence of diabetes(99;175;177;186). In one study, weight gain of > 5 kg was associated with higher rate of progression of fibrosis(98). Other variables that have been independently associated with hepatic fibrosis in some studies have been age(173;177;186), male gender(176), hypertension(176) and triglyceride levels(177).

The role of cytokines in the “second hit” of NAFLD has been described in Chapter 1.3. Epidemiologically, there is cross-sectional evidence linking higher levels of cytokines with

progression of NAFLD. TNF $\alpha$  levels have been shown to be higher in patients with NASH compared to those with simple steatosis, and NAFLD compared to non-steatotic controls (187-192). In some studies this relationship was independent of confounders, but in others adjustment for markers of insulin resistance and adiposity abolished the relationship(188). A statistically significant relationship of TNF $\alpha$  to hepatic fibrosis has also been observed(188). Studies that have not shown a statistically significant relationship between TNF $\alpha$  and NAFLD severity have often been small or there has been a trend towards an association(193;194). In one study of obese patients in which TNF $\alpha$  levels were similar across groups of increasing liver disease severity, it is possible that medication use blurred distinction between the groups(195). Results for IL-6 have generally paralleled those for TNF $\alpha$ (188;190), with a stepwise increase in IL-6 concentration through groups with normal liver, simple steatosis and NASH(59). In other studies the association of plasma IL-6 with stage of liver disease has been weaker(188;191).

#### *Histological risk factors for hepatic fibrosis*

In a number of cross-sectional studies the presence of significant fibrosis has been positively associated with the severity of steatosis and necroinflammatory change, but this has not been a universal finding. Results from a study of obese individuals demonstrated age, degree of steatosis and grade of inflammation to be the only factors independently associated with stage of fibrosis(126); similarly, in another obese population, necroinflammatory activity was strongly associated with the presence of septal fibrosis(177). This has not, however, been a universal finding(180). In longitudinal studies the relationship between baseline histology and progression of fibrosis has been similarly inconsistent. In one study, with a moderate prevalence of simple steatosis (rather than more advanced liver disease) at baseline, those who progressed had significantly more pronounced steatosis at baseline, whereas necroinflammatory changes were unrelated to progression(98). In contrast, a study which looked at clinical outcome as related to baseline biopsy findings found that the presence of features of inflammation, necrosis or fibrosis was related to a significantly higher incidence of cirrhosis over a mean period of 8.2 years when compared to steatosis alone (22% vs 4%)(67). A further study showed only fibrosis stage at baseline to be independently (and inversely) associated with fibrosis progression, whereas baseline severity or subsequent worsening of steatosis or NASH were not predictive(99). Although the inverse relationship between fibrosis at baseline and subsequent worsening of liver disease severity

appears counterintuitive, it can be at least partly explained by the fact that those with the worst liver disease at baseline have less scope for further progression.

### **2.4.3 Clinical risk scores in the prediction of hepatic fibrosis**

In the absence of an easily-utilised biochemical test for fibrosis several research groups have constructed clinical risk scoring systems through statistical modelling, which combine routinely-available, and therefore inexpensive, physical and biochemical characteristics. Many of these have been discussed above. A key aim of such scores is to accurately define patients who do *not* have advanced fibrosis, with the aim of avoiding unnecessary biopsy: a high sensitivity and NPV is therefore important in excluding fibrosis with accuracy.

The BAAT score was developed in an overweight population ( $BMI > 25 \text{ kg/m}^2$ ) and assigned one point for each of increased age, BMI, ALT and triglycerides(177). Using a positive score of 2 or above to denote the presence of septal fibrosis, the PPV was 45% and NPV 100%; a score of 3 or above had a PPV of 100% and a NPV of 70-73%. The BARD score allocated one point for each of increased BMI and the presence of diabetes, and two points for AST:ALT ratio greater than 0.8(196). With a cut-off of 2 points or more, the PPV and NPV of determining severe fibrosis (Stage 3 – 4 on the Brunt staging system) were 43% and 96% respectively. In a validation study within a UK cohort, the PPV was 27% and NPV 95%(197). The NAFLD fibrosis score uses a formula combining age, BMI, the presence of diabetes, AST:ALT ratio, albumin and platelet levels to classify participants as low, indeterminate or high risk for severe (septal or bridging, stage 3 – 4 on the Brunt staging system) fibrosis(198). The PPV of a high risk score was 90% in the estimation group and 82% in the validation group; the NPV of a low risk score was 93% in the estimation group and 88% in the validation group. The authors conjectures that biopsy could be avoided in the 75% of participants calculated to be low or high risk, and used mainly in those that were scored as indeterminate. This score has been further validated in UK, Argentinian and Chinese populations with similar positive and negative predictive values(197;199;200).

By definition, these scoring systems have been developed and validated in tertiary referral centres on a selected group of patients in whom biopsy has been felt to be clinically necessary and in whom elevated liver enzyme levels was often a prerequisite for

investigation. One must be wary that an association found in patients at the severe end of the clinical spectrum may be less relevant to those with less severe liver disease. Furthermore, the PPV and NPV values quoted are by definition dependent on the prevalence of advanced liver disease in the population studied. No scoring system has been created for use specifically within populations of patients with diabetes and, interestingly, when the BARD score was examined in the diabetic subgroup of the original study population it was found to be less predictive than in the subgroup without diabetes (ROC AUC 0.53 vs 0.80).



## **2.5 Outcome of NAFLD – morbidity and mortality**

Several studies have examined the longterm outcome of NAFLD in general populations, in terms of clinically appreciable endpoints – cirrhosis-related complications, HCC and death. There is also a considerable body of evidence demonstrating the relationship of diabetes to these hard outcomes, suggesting a deleterious effect when the pathologies co-exist.

### **2.5.1 Morbidity – HCC and other complications of cirrhosis**

Early studies examining the aetiology of liver disease in registers of patients with proven HCC found underlying cryptogenic cirrhosis in 7 – 29%, and features of NAFLD in 13%(201;202). These established NAFLD (both with and without cirrhosis) as a possible cause of HCC and prompted studies into its incidence within populations of patients with NAFLD. Incidence of HCC has been estimated to be 2.4% over a maximum follow-up period of 21 years of patients with biopsy-proven NASH(178), 1.6% over 13 years in a study of biopsy-proven NAFLD(98), and 0.5% over 7.6 years in a study of NAFLD diagnosed on imaging or biopsy(203). In other longitudinal studies, encompassing groups with steatosis alone and NASH, no cases of HCC were diagnosed over follow-up times of 3 -11 years(99;179-181). In a study of patients with biopsy-proven NAFLD, 5.4% developed cirrhosis-related complications, including ascites, HCC and variceal haemorrhage, over 13 years of follow-up(98).

### **2.5.2 Mortality**

The majority of studies to examine the relationship of NAFLD with mortality have found NAFLD to be associated with a higher mortality rate. A population-based cohort study, in which patients with NAFLD diagnosed on imaging or biopsy were compared to the age- and sex-matched general population, found significantly lower survival in the NAFLD group with a standardised mortality ratio of 1.34(203). On multivariate analysis, only age, the presence of cirrhosis and the presence of impaired glucose tolerance or diabetes were independently associated with rate of death. In a second study, of patients with biopsy-proven NAFLD in the context of elevated LFTs, survival over a mean of 13 years was significantly lower than in the corresponding reference population(98).

Conversely, in two studies originating from the NHANES cohort no relationship between NAFLD and mortality was found. The first was a study comparing patients with elevated ALT with those with a normal ALT level, after exclusion of people with alcohol excess, viral hepatitis, iron overload and hepatotoxic medication use(204). There was a trend towards higher age-matched all-cause mortality rate in the group with high ALT levels but this did not reach statistical significance. This study had the advantage of a relatively non-selected patient population, but the disadvantage of the use of a surrogate marker to designate NAFLD. Interestingly, when data from the same study was analysed using higher cut-offs to define elevation in ALT, looking at a slightly different age-range of participants and performing multivariate adjustment for confounding variables, the overall and liver-related mortality was found to be unquestionably higher in the NAFLD group(205). The second study used ultrasound (with the same exclusions on the basis of secondary causes for liver disease as above) to diagnose NAFLD, and found no evidence of an association between NAFLD and all-cause mortality or deaths due to cardiovascular or liver disease(206). It is possible that in this unselected population the majority of participants with NAFLD had simple steatosis, which has been suggested to be of lower risk than more aggressive forms of liver disease as described below. However, if ultrasound measures were not robust, or if a proportion of the control group actually had hepatic fibrosis (which would mask the finding of hepatic steatosis on ultrasound) then a false negative result is possible.

As with biopsy-diagnosed progression of liver disease, there is evidence that mortality is related to baseline histology. In a study comparing outcome of patients with NASH with those with simple steatosis alone, there was a trend towards an increase in liver-related death in the NASH group but this did not reach statistical significance (10% vs 2%,  $p = 0.08$ )(67). Compared to the age-matched general population, however, there appeared to be a very significantly elevated risk of liver-related death in the NASH group. This was mirrored in another cohort of patients with NAFLD.(98). In a further study, advanced (bridging or cirrhosis) fibrosis was the only histological parameter to be significantly associated with liver-related mortality, with an adjusted hazard ratio of 5.68(207). The relatively better prognosis in simple steatosis has also been indicated in a study of subjects with fatty liver alone, in which the survival rate was found to be similar to the general population, with a liver-related mortality of 1% over 16.7 years(208).

Although the data regarding the effect of NAFLD on mortality is not absolutely uniform, the conclusion that NAFLD is associated with reduced survival appears relatively well-founded. Results from different studies depend on method of ascertainment of NAFLD and baseline histology. In cohort studies examining subjects with pre-diagnosed NAFLD there is the possibility of referral bias – for example, referral may have been more likely in the context of adverse metabolic features such as diabetes or significant obesity that may in themselves affect outcome. It has been demonstrated that subjects that undergo liver biopsy have a poorer prognosis than those that do not, and this is likely due to a higher presence of indicators of significant liver disease in these patients(203). Examination of the long-term outcome in unselected patient populations, assessed reliably at baseline for the presence of NAFLD, would go some way towards overcoming these limitations.

### **2.5.3 Poor outcome with the combination of NAFLD and Type 2 diabetes**

The relationship of diabetes to clinically apparent chronic liver disease and mortality has been explored in a number of studies. A large cohort study was designed to investigate a causal role of diabetes in promoting increased severity of liver disease. This study followed 173643 mainly male Veterans with diabetes and 650620 controls for 10-15 years(209). The presence of a diagnosis of liver disease at baseline was an exclusion criterion for this study, thus reducing the impact of diabetes that has arisen secondary to cirrhosis. There was a significantly higher incidence of chronic liver disease in the group with diabetes compared to that without diabetes (incidence rates 18.13 per 10000 person-years and 9.55 per 10000 person-years respectively, with a relative risk among those with diabetes of 1.98). When patients with a new diagnosis of hepatitis B or C, alcohol use or alcoholic liver disease were excluded the relative risk of chronic liver disease in the group with diabetes was 2.15. The main limitation of this study was failure to formally seek undiagnosed subclinical liver disease at baseline and, given the indolent nature of NAFLD, it is possible that a proportion of patients would already have had evidence of this condition which would detract from conclusions regarding temporal relationship. Misclassification of a number of patients on the basis of errors of coding is likely in a study of this magnitude, but is unlikely to have materially affected the outcome.

Diabetes is an established risk factor for HCC and in one population-based study, diabetes was associated with around a 2.5-fold increase in incidence of HCC, independent of alcohol

intake and viral hepatitis(209). This has been mirrored in another similarly large population study, with reported standardised incidence ratios of primary liver cancer within patients diagnosed with diabetes of 2.1 in women and 4.0 in men. Secondary causes of liver disease were not excluded in this study(210). The prevalence and incidence of HCC in the general population of people with Type 2 diabetes, however, remains unclear, with no cases reported in NAFLD prevalence studies within Type 2 diabetic populations(18;170;173).

The presence of diabetes has been an independent predictor of mortality in studies outlined above that have examined the impact of NAFLD(203;205). These studies have not, however, been able to fully elucidate the pathogenic processes underlying this, and in particular it is unclear what proportion of the effect of diabetes is via increased progression of liver disease versus more established sequelae such as cardiovascular disease and malignancy. In contrast, in one population-based study investigated death from liver cirrhosis and found the risk to be 2.5 times higher in people with type 2 diabetes compared with the general population(211). Information on secondary causes of liver disease was not available.

The effect of NAFLD on mortality within a population of patients with a prior diagnosis of Type 2 diabetes has also been examined(212). In this population NAFLD, after adjustment for age, gender, duration of diabetes, obesity and other cardiovascular risk factors, was associated with a significantly increased risk of death compared to a control group, with a hazard ratio of 2.2. When different causes of death were examined, NAFLD was associated with a borderline increased risk of death from malignancy but there was no trend towards increased mortality due to cardiovascular disease. Nineteen percent of NAFLD patients died from liver-related causes (liver failure, HCC and variceal bleeding), compared with no patients in the control group.

## 2.6 Treatment of NAFLD

Treatment of NAFLD involves a multi-faceted approach, in much the same way as is utilised in the treatment of diabetes. There is considerable overlap in the possible management strategies used in the two conditions, particularly as proposed treatments for NAFLD have often focused on improving insulin resistance, also a key target in Type 2 diabetes. Currently, specific pharmacological interventions for NAFLD (specifically NASH) are supported by relatively limited evidence, and are considered to be experimental and the subject of ongoing research. Evidence for benefit in terms of “hard outcomes” such as liver failure and mortality is lacking. The absence of a definitive treatment is one reason why patients with Type 2 diabetes are not routinely comprehensively screened for NAFLD, and there is a perception that making the diagnosis will not change management in this patient group; concomitant with this is the incomplete knowledge of the natural history of the disease and the best strategy for following up those who are found to have NAFLD. Currently, identification of those with NASH might allow their inclusion in clinical trials of newer agents. Of most importance, however, is recognition of individuals that have (or are at significant risk of developing) cirrhosis, as screening for oesophageal varices and HCC is recommended in this group(213). Liver transplantation remains an option for some people with end-stage liver disease, and NAFLD accounts for over 10% of people on the transplant waiting list in the UK(8).

A full review of the treatments that have been trialled in NAFLD is beyond the scope of this review, but below is mention of the most prominent with particular reference to those that target insulin resistance.

### 2.6.1 Treatments that target insulin resistance

#### *Lifestyle modification*

The role of lifestyle change, specifically with the aim of weight reduction and improved insulin sensitivity via diet and exercise interventions, has recently been the subject of a systematic review(214). Many of the trials included were small or uncontrolled, and few had included serial biopsies, with most following participants using imaging or aminotransferase levels. Almost all studies reported reductions in hepatic fat or liver enzyme levels, often in

parallel with weight loss. A randomised controlled study has examined the effect of a lifestyle intervention, including a combination of diet, exercise and behaviour modification, on liver histology in individuals with biopsy-proven NASH(141). Over the 48-week trial period there was a reduction in body weight of 9.3% in the intervention group compared to 0.2% in the control group, and this was accompanied by a significantly greater improvement in NASH clinical activity score. When individual histological features were examined, there was a significantly greater improvement in the steatosis score in the intervention group, ballooning injury score improved to a similar extent in both groups, and fibrosis score was unchanged. Changes in histology were significantly correlated with extent of weight loss in both groups, and subjects who attained 7% weight loss had significantly greater reductions in steatosis, lobular inflammation and ballooning injury scores. In general the evidence for lifestyle modification suggests that moderate, controlled weight loss should be promoted in patients with NAFLD with the aim of improving steatosis and inflammatory changes; the evidence for improvement in more advanced liver disease is currently lacking.

### *Metformin*

Metformin is very commonly used as an insulin-sensitizing agent in the treatment of Type 2 diabetes. However, despite an initial trial which suggested an improvement in aminotransferase levels with metformin treatment, direct evidence for benefit in NAFLD is lacking(215). In four high-quality randomised controlled trials in which serial biopsies were taken, 6-12 months treatment with metformin plus lifestyle intervention did not improve liver histology over lifestyle alone(216).

### *Thiazolidinediones*

In terms of pharmacological modification of the course of NAFLD, the thiazolidinediones (peroxisome proliferator-activated receptor  $\gamma$  agonists) have most evidence for efficacy. Their mechanism of action is to ameliorate insulin resistance by redistributing fat from the liver and muscle to peripheral adipose tissue. The pooled results of five high quality randomised controlled trials of treatment with thiazolidinediones, in which serial biopsies were undertaken, have shown improvement in histological steatosis and inflammation but not fibrosis(216). In the context of Type 2 diabetes, the one study to specifically recruit participants on the basis of impaired glucose tolerance or diabetes is of particular

interest(217). In this placebo-controlled study, after 6 months of treatment, there were significant improvements in steatosis, ballooning necrosis and inflammation, and a trend towards a significant reduction in fibrosis ( $p = 0.08$ ). In a larger study of non-diabetic patients, comparison of pioglitazone therapy with placebo did not meet the pre-specified level of significance for the primary outcome, which required an improvement of one or more points in the hepatocyte ballooning score, no increase in the fibrosis score and a decrease in the NAFLD activity score of at least 2 points or to a final score of 3 or less(218)s. The pioglitazone group, however, had fewer participants with hepatocyte ballooning at baseline, and this may have contributed to the negative finding of this primary endpoint. Pioglitazone was superior to placebo in secondary endpoints of reduction in steatosis, lobular inflammation and the NAFLD activity score.

### **2.6.2 Other treatments**

The treatments outlined above have focused on insulin resistance as a target; an alternative strategy is to specifically target oxidative stress and inflammation, which are thought to play a role in the “second hit” of NAFLD. A meta-analysis of antioxidants found no histological benefit, but there was significant heterogeneity between studies, in particular with regard to the drug administered and treatment duration(216). A two-year randomised placebo-controlled trial of Vitamin E has stimulated interest, as Vitamin E was associated with a significantly higher rate of improvement of NASH compared with placebo, and on analysis of the separate components of severity showed improvement in steatosis, lobular inflammation and hepatocellular ballooning (but not fibrosis)(218). There are, however, concerns regarding long-term safety of Vitamin E as it has been associated with an increased risk of cardiovascular disease and haemorrhagic stroke(219). The anti-TNF $\alpha$  agent, pentoxifylline, has also been associated with improvements in liver histology (steatosis and hepatocellular ballooning) compared with baseline in a small study, but outcome compared to placebo failed to show a statistically significant difference(220).

## **2.7 Relationship of NAFLD with cardiovascular disease**

The relationship between NAFLD and cardiovascular disease has been explored both in populations of people with Type 2 diabetes and those without. As described below, a positive relationship between NAFLD has been found with hard clinical endpoints including myocardial infarction, stroke and cardiovascular death, and with markers of subclinical cardiovascular disease.

### **2.7.1 Association of NAFLD and cardiovascular events**

In a prospective study of over 1600 Japanese employees, the presence of NAFLD diagnosed on ultrasound at baseline was associated with an increased 5-year incidence of cardiovascular events (5.2% vs 1.0%,  $p < 0.001$ )(221). This association persisted after adjustment for traditional cardiovascular risk factors and features of the metabolic syndrome. Within a population of patients with Type 2 diabetes, 248 participants who suffered non-fatal coronary heart disease, stroke or cardiovascular death over a five-year period were age- and gender-matched with control subjects who remained free of cardiovascular disease(222). The group that developed cardiovascular disease had a significantly higher prevalence of ultrasound-diagnosed NAFLD at baseline (94% vs 56%,  $p < 0.001$ ). The univariate association persisted after adjustment for classical cardiovascular risk factors, diabetes duration, glycaemic control and medication use(7;222). Adjustment for features of the metabolic syndrome, including hypertension, attenuated but did not abolish the significant association. These results have been supported by cross-sectional studies in patients with Type 2 diabetes that have found similar associations(18;223).

### **2.7.2 Association of NAFLD and surrogate markers of cardiovascular disease**

#### *Coronary atherosclerosis*

NAFLD has been associated with increased prevalence of coronary artery disease. In a study of Chinese patients undergoing diagnostic coronary angiography, 84.6% of those with NAFLD on ultrasound had a coronary stenosis of  $> 50\%$ , compared to 64.1% of those with no evidence of fatty liver(224). This association remained after adjustment for the other factors that had a statistically significant association on univariate analysis (age, gender, diabetes, WC, fasting glucose, high density lipoprotein (HDL) cholesterol and ALT); but



unfortunately not including smoking or blood pressure, and there was no measure of insulin resistance). In the same study, in patients with proven established coronary artery disease, there was no association between NAFLD and a composite endpoint including cardiovascular death, non-fatal MI and need for further coronary intervention over a time period of up to 120 weeks.

#### *Carotid intima media thickness*

NAFLD has been associated with an increased risk of carotid atherosclerosis, as defined using carotid intima media thickness (CIMT) measurement, a well-validated screening tool for the prediction of cardiovascular disease in asymptomatic subjects. In a study of 40 patients with USS-diagnosed NAFLD, in whom those with a history of alcohol excess, viral hepatitis and high transferrin saturation were excluded, and the same number of age- and gender-matched controls in whom hepatic steatosis was not found on USS, NAFLD was found to be associated with a significantly higher CIMT and this persisted after adjustment for traditional cardiovascular risk factors, the presence of the metabolic syndrome and insulin resistance as measured using the HOMA-IR score(225). A further study of non-diabetic patients with NASH, an age- and gender-matched group with chronic viral hepatitis and a further group of controls who had normal liver enzymes and ultrasonography observed a statistically significant stepwise increase in CIMT through the normal, viral hepatitis and NASH groups(226). The relationship between NASH and CIMT remained after adjustment for traditional cardiovascular risk factors and features of the metabolic syndrome including insulin resistance as measured using the HOMA-IR score. Similar results have been found in other study populations including children with NAFLD(227;228). In an additional study in non-diabetic men the positive association between the presence of NAFLD and CIMT was independent of insulin resistance as measured by HOMA-IR and WC, but abolished by the inclusion of visceral abdominal fat as measured on CT cross-sectional imaging, raising the possibility that visceral fat accumulation is a key component of the relationship between NAFLD and atherosclerosis(229). The main study to examine the relationship between the different pathological stages of NAFLD found that CIMT was significantly positively associated with the presence of NASH over fatty liver alone, and that there were stepwise increases in mean CIMT through increasing grades and stages of steatosis, inflammation and fibrosis(230). These associations persisted after adjustment for traditional cardiovascular risk factors, insulin resistance as defined by the HOMA-IR score and features of the metabolic syndrome.

Interestingly, in populations of patients with Type 2 diabetes the association of NAFLD with CIMT has been more variable. In one study of Type 2 diabetic patients, in which hepatic steatosis was quantified using MRS, there was no difference in CIMT in participants with, versus those without, NAFLD(231). However, there was no attempt made to adjust for confounding factors such as gender or medication use (there was a significantly higher rate of metformin use in the NAFLD group). In two other studies of patients predominantly with Type 2 diabetes there was a significantly higher CIMT in the group with hepatic steatosis or NAFLD, but this relationship was abolished after adjustment for other variables, in one case specifically insulin resistance as measured using the HOMA-IR score(228;232). One possible reason for the variability in results is that the bias in selecting diabetic patients, who are by definition at high cardiovascular risk and are likely to have higher CIMT measurements than the general population, makes it more difficult to demonstrate independent relationships between NAFLD and CIMT. It is also possible that misclassification of patients on the basis of ultrasound grading of steatosis has contributed to negative results, but this would be less likely in the case of MRS being used to define steatosis. It is, however, also possible that the effect of NAFLD on cardiovascular risk is not principally mediated by through chronic atherosclerosis, or indeed that NAFLD is not a direct mediator of increased cardiovascular risk in the diabetic population but instead an epiphenomenon.

### *Endothelial dysfunction*

Another surrogate marker of early atherosclerotic disease, endothelial function as measured by flow-mediated vasodilatation of the brachial artery, has been found to be associated with NAFLD. In a study of NAFLD cases and matched controls the (endothelium-dependent) vasodilatation of the brachial artery, in response to reactive hyperaemia in the context of an inflated then deflated blood pressure cuff, was found to be significantly less in the NAFLD group(233). This was independent of age, gender, BMI and other features of the metabolic syndrome. Similar results were found in a study of children with NAFLD, when compared to obese controls(227). Although the working hypothesis was that endothelial dysfunction was a marker of atherosclerosis, it cannot be excluded that liver disease has its own direct effect on the endothelium.

### **2.7.3 Conclusion**

An association between NAFLD and cardiovascular disease now seems established, with the main area of controversy whether this relationship is independent of insulin resistance. Several other non-classical risk factors that might implicate a causal role for NAFLD have been explored, including reductions in circulating levels of the cardioprotective cytokine adiponectin, and abnormal lipoprotein metabolism that leads to a pro-atherogenic profile with overproduction of triglyceride- and cholesterol-rich remnant particles(234). In the next section the focus will be on two pathways that may link the two conditions – a pro-thrombotic tendency related to increased production of pro-thrombotic mediators, and a systemic subclinical inflammatory state.

## **2.8 Non-traditional mediators of NAFLD-associated cardiovascular risk – clot kinetics and pro-thrombotic mediators**

### **2.8.1 Clot kinetics and prothrombotic mediators**

#### *Pathways of clot formation and lysis*

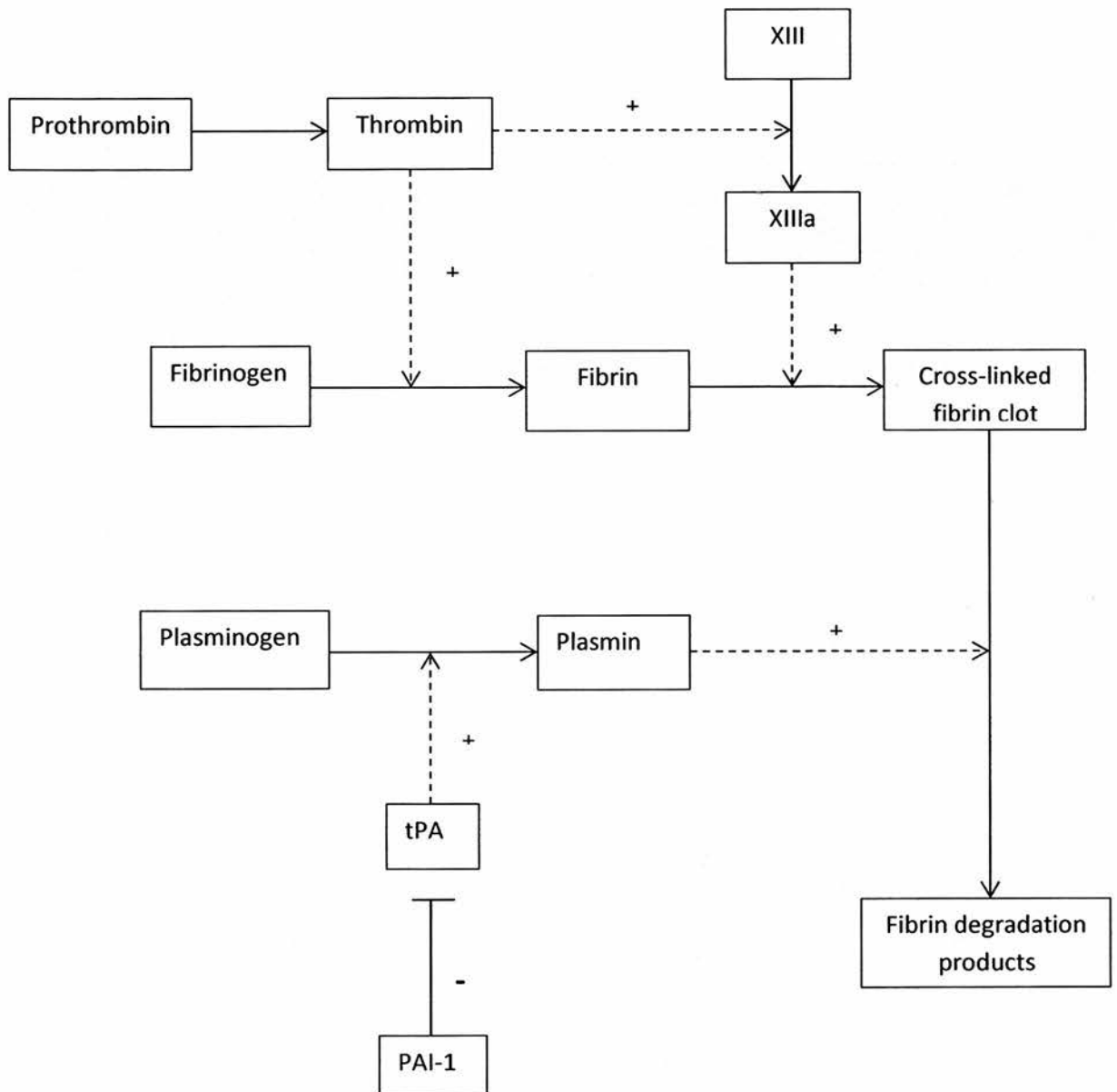
Formation of occlusive thrombus at the site of rupture or erosion of an atherosclerotic plaque is the final step in acute vascular events such as myocardial infarction(235). Clot formation involves a complex interaction between coagulation factors (synthesised by hepatocytes) and platelets that culminates in a common pathway of thrombin formation and fibrinogen activation, summarised below in Figure 2.1(236). Fibrinogen is cleaved by thrombin, enabling interaction between fibrin molecules and the development of a cross-linked fibrin clot. This clot is additionally stabilised by the incorporation of activated Factor XIII, which further cross-links the fibrin molecules.

Conversely, the fibrinolytic system mediates the lysis of cross-linked fibrin clot and this process is again encapsulated in Figure 2.1 below. The initial step in this pathway is production of tissue plasminogen activator (tPA) from endothelial cells and this molecule subsequently activates plasminogen to plasmin, which in turn breaks down the fibrin structure of the thrombus. Plasminogen activator inhibitor type 1 (PAI-1) is a physiological inhibitor of this process, by binding to and inactivating tPA.

There is a finely-balanced equilibrium between coagulation and fibrinolysis in health. This can be disturbed by subtle perturbations in concentration and function of the components of these systems. A hypercoagulable state has been linked to increased cardiovascular risk, and the evidence behind this is discussed in subsequent sections.

Figure 2.1

Formation and lysis pathways of the fibrin-rich clot.



tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor type 1

### *Association between clot dynamics and cardiovascular disease risk*

An area of particular interest is the effect of inter-individual differences in clot structure and propensity to lysis on cardiovascular risk. Research in this field has been enabled by the development of techniques that allow observation of changes in clot density over time *in vitro*, including turbidimetric assays that measure scatter of light by solid particles (clot turbidity having been shown to be directly proportional to the cross-sectional area of the fibrin fibres)(237), and thromboelastography which assesses viscoelastic changes during clot formation and breakdown(238). In addition, confocal and electron scanning microscopy has allowed detailed assessment of clot microstructure.

It has been observed that individuals at high cardiovascular risk have higher clot density, altered clot morphology and longer clot lysis times compared with controls(239-242). In a study comparing 33 young post-myocardial infarction patients with age- and sex-matched healthy controls, clots from the post-myocardial infarction patients had a more compact architecture - with thinner, more numerous fibrin fibres and smaller pores - than the controls(241). This was paralleled by resistance to clot lysis in these patients, with independent predictors of clot lysis time including fibrin fibre length and stiffness, and concentration of PAI-1. A less porous clot structure has also been observed in healthy relatives of patients with coronary artery disease, in whom examination could be performed in the absence of confounding factors such as ubiquitous aspirin use(240).

Similar alterations in clot structure and lysis times have also been seen in individuals with the metabolic syndrome or diabetes(239;243-245). Clots formed using purified fibrinogen from patients with Type 2 diabetes have been shown to be more dense when compared to those formed from the fibrinogen of age- and sex-matched controls, with a positive association between worsening glycaemic control and less permeable clot structure(244). Similarly, the presence of the metabolic syndrome has been associated with denser clots and longer clot lysis times compared to controls, and there was a progressive stepwise increase in these variables though increasing numbers of metabolic syndrome components(239). In a different study, clot lysis times were longer in children with Type 1 diabetes than in controls and were significantly shorter after improvement of glycaemic control(243). The results from these studies suggest that part of the increased cardiovascular risk associated with

insulin resistance, diabetes and poor glycaemic control is mediated through alteration in clot formation and lysis kinetics.

*Association of pro-thrombotic mediators, clot kinetic variables and cardiovascular disease*

Many of the above studies have also examined the relationship of plasma levels of prothrombotic mediators with clot formation and lysis kinetics. Fibrinogen concentration and structure, as might have been expected, has been particularly strongly associated with clot density measures but has also been shown to contribute to variation in clot lysis(239;240;246;247). PAI-1 contributed 7-13% of variation in clot lysis in one study, and was related to fibrinolysis rate in further reports(239;241;243;248). Administration of an antibody to PAI-1 resulted in a doubling of tPA-induced thrombolysis in one experimental model(249).

The distinctions between pro-thrombotic and pro-inflammatory mediators has become blurred in recent times and it is now recognised that there is considerable overlap between the two systems. Thus, fibrinogen and PAI-1 have been acknowledged as acute phase proteins, while complement C3, a central component of the innate immune system, has been shown to play a role in clot formation. In a study performed in children with Type 1 diabetes and age-, sex- and weight-matched controls, both C3 and C-reactive protein (CRP) were higher in the diabetic group, but only C3 level correlated with clot lysis variables(243). C3 was shown to be incorporated into clots with subsequent prolongation of clot lysis time. The association between C3 concentration and clot lysis time has been confirmed in further studies in adults(250;251).

The associations of plasma levels of fibrinogen, PAI-1 and C3 with clot formation and lysis dynamics have been mirrored by their relationship with clinically meaningful cardiovascular endpoints. Fibrinogen has been the most extensively studied. An early meta-analysis found a positive association between fibrinogen concentration and incident cardiovascular disease in all seven studies that had previously examined that relationship, with an overall odds ratio of 2.3 (95% confidence intervals 1.9 – 2.8) for upper versus lower tertiles of fibrinogen(252). This effect persisted after adjustment for established confounders such as age, smoking, BP

and lipid measures, but studies were inconsistent in their inclusion of measures of obesity and glycaemia. Furthermore, pre-existing cardiovascular disease was not always an exclusion criterion, and therefore reverse causation could not be discounted. The positive association of fibrinogen with subsequent cardiovascular events was confirmed in a later and larger meta-analysis of 31 studies including over 154000 participants in total, in which a hazard ratio of 2.4 (95% confidence intervals 2.2 – 2.6) in predicting coronary heart disease was observed for each 1 g/l increase in fibrinogen(253). This report attempted to address the limitations inherent in the earlier meta-analysis: only subjects in whom there was no history of clinically apparent coronary heart disease or stroke were included (although attempts were not made to exclude subclinical atherosclerosis); and, when possible, a full adjustment for other cardiovascular risk factors (including BMI and the presence of diabetes) was performed. After such an adjustment the hazard ratio was 1.8 (95% confidence intervals 1.6 – 2.1). Similar results have been seen in populations of patients with Type 2 diabetes(254;255). Despite the consistent associations between higher fibrinogen levels and increased incidence of cardiovascular disease in epidemiological studies, a firm association is yet to be shown between genetic determinants of higher fibrinogen levels and subsequent risk of cardiovascular disease(256-258). This raises the possibility that fibrinogen does not play a direct role in the pathogenesis of cardiovascular disease, but instead may be a marker for other causal factors. An alternative explanation is that environmental influences on fibrinogen level are more potent than genetic ones, or indeed that higher fibrinogen levels are a product of the interaction between genetic and environmental effects.

The relationship of PAI-1 to cardiovascular disease has similarly been investigated, but results have been rather less uniform. One early study suggested an independent association between PAI-1 and myocardial re-infarction in 109 survivors of first myocardial infarction who were followed for three years(259). Higher PAI-1 at baseline remained an independent predictor after adjustment for traditional cardiovascular risk factors, angiographic findings and in-depth measures of glucose tolerance and insulin sensitivity. A later study carried out in 147 patients with Type 2 diabetes showed a significant cross-sectional relationship between PAI-1 and coronary heart disease that was unattenuated by adjustment for traditional risk factors, the metabolic syndrome and diabetes variables(260). In contrast, other studies have observed a trend only towards higher PAI-1 levels in participants that go on to have a cardiovascular event(261), or an initially significant association that is fully attenuated by adjustment for traditional and metabolic risk factors(262-264). A meta-analysis



of studies that examined the effect of PAI-1 gene polymorphisms (specifically the 4G polymorphism within the promotor region that has been associated with approximately 20% higher PAI-1 levels) found weak associations between this polymorphism and myocardial infarction (relative risk 1.04 (1.00 – 1.09), but the strength of the findings was reduced by concern over publication bias(265). In summary, there is fairly consistent evidence for a positive association between PAI-1 and cardiovascular disease, but variable support for its independence of other metabolic variables, calling into question the theory that it has a direct causative role.

The relationship of complement C3 to cardiovascular events has been the subject of fewer reports than that of fibrinogen and PAI. In one study of 860 participants within an Italian population, C3 levels at baseline were shown to be independently related to incident ischaemic events including any form of coronary heart disease and myocardial infarction after adjustment for traditional risk factors and obesity(266). An independent association was also found in another study of 1200 middle-aged and elderly participants, in which relative risk of coronary heart disease for each standard deviation increase in C3 was 1.35 (95% confidence intervals 1.09 – 1.67) after adjustment for age, sex, presence of the metabolic syndrome, smoking status and CRP amongst others(267). In contrast, other authors have observed complete attenuation of the relationship of C3 and coronary events following adjustment for established cardiovascular risk factors(268). Adjustment for direct measures of insulin resistance was not carried out in any of these studies.

#### *Relationship of NAFLD with clot formation and lysis dynamics, and components of the coagulation cascade and fibrinolytic system*

The theory that the relationship of NAFLD with cardiovascular disease may be in part mediated by altered clot formation and lysis dynamics and a pro-thrombotic state has been based on a number of considerations. The liver, as the primary site of production of most components of the coagulation and fibrinolytic systems, is integral to the clotting cascade. Furthermore there is evidence to suggest that hepatic production of some pro-thrombotic mediators, such as PAI-1, is enhanced in response to liver injury(269). As has been outlined above, hepatic steatosis is intrinsically bound up with the subclinical inflammatory state that accompanies visceral adiposity and insulin resistance, and this leads to a vicious circle of pro-inflammatory cytokine release and upregulation of pro-thrombotic mediators(270-274).

Thus, NAFLD may modulate cardiovascular risk directly via hepatic synthesis of these mediators, but may also be representative of a high-risk environment in which adipose tissue also plays a role.

To date, investigation of clot dynamics in patients with NAFLD has been limited to one small study. This analysis compared clotting kinetics, measured using thromboelastography, in 28 patients with NAFLD (either diagnosed on ultrasound or on biopsy) and 22 control subjects(275). A higher clot strength and reduced clot lysis was demonstrated in the group with NAFLD, independent of the presence of diabetes or features of the metabolic syndrome.

Although a robust relationship between pro-thrombotic mediators and insulin resistance has been demonstrated(276;277) there have been conflicting results when associations with liver disease have been examined. The steatotic liver has been directly implicated in increased production of PAI by a small histological study, in which hepatocyte staining for PAI-1 protein was significantly greater in the NAFLD versus the control group(278). These results were supported by the finding of a significant relationship between liver histology in NAFLD and circulating PAI-1 levels that persisted after adjusting for insulin resistance and features of the metabolic syndrome(279). A large study of over 500 subjects revealed an association between ALT and C3 levels which persisted after adjustment for insulin resistance(277). In contrast, two studies have observed complete attenuation of the relationship between hepatic steatosis and higher fibrinogen and PAI-1 levels after adjustment for components of the metabolic syndrome(274;280). The relationship of these pro-thrombotic mediators to clot kinetics has been little studied in relation to NAFLD in human subjects and not at all in NAFLD in the context of type 2 diabetes.

## Chapter 3

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### Research Aims

#### 3.1 Research Aims

The following chapters describe work undertaken within the Edinburgh Type 2 Diabetes study with the aim of enhancing understanding of the prevalence and epidemiology of NAFLD within a large population of people with Type 2 diabetes.

##### 3.1.1 Use of ultrasound to diagnose hepatic steatosis

Ultrasound imaging is an established modality in the diagnosis of hepatic steatosis, both clinically and in large-scale studies. However, diagnostic accuracy has varied between studies and a degree of subjectivity in ultrasound grading of steatosis is well-recognised. Despite this, most studies that rely on an ultrasound measure for steatosis have not had this measure validated, and formal examination of intra- and inter-observer variation of ultrasound grading has been infrequently performed.

Our primary aim was to validate our ultrasound measure of steatosis in a subgroup of participants, by comparing ultrasound gradings with hepatic fat fraction on MRS, the non-invasive gold standard measure of steatosis. In so doing, we intended not only to substantiate the measure on which the rest of our study depends, but also to add to the literature describing the accuracy of ultrasound by performing the first comparison with MRS. This is particularly valid as biopsy (the accepted gold standard) is imperfect and in particular samples a tiny proportion of the liver as a whole, and significantly less than either MRS or ultrasound.

A secondary aim was to investigate intra- and inter-observer variability in ultrasound grading between three graders: an ultrasonographer, who was responsible for scanning all study

participants; a specialist registrar in radiology; and a consultant radiologist. Thus we aimed to provide a measure of variability that encompassed “real life” variation in graders.

### **3.1.2 Prevalence of and risk factors for hepatic steatosis and NAFLD in Type 2 diabetes**

Despite the established pathophysiological and epidemiological associations between Type 2 diabetes and NAFLD, few studies have examined the prevalence of NAFLD in diabetic populations. Those that exist have often focused on small, hospital-based cohorts, or have used an unvalidated ultrasound measure or incomplete exclusion of secondary causes for liver disease. Screening for liver disease in people with Type 2 diabetes routinely involves performing liver enzyme measurements. Studies performed in the general population have suggested that these biochemical markers are poorly sensitive in identifying fatty liver, but there are few data from within populations of patients with Type 2 diabetes looking at this issue.

The main aim of the study was to accurately determine the prevalence of hepatic steatosis and NAFLD in a large, unselected population of older people with Type 2 diabetes, using a MRS-validated ultrasound measure and strict exclusion of secondary causes for liver disease. It is anticipated that the results will have public health implications in estimating the number of patients that might require monitoring for more advanced liver disease, and in preliminary identification of those that may in the future benefit from disease-modifying agents. Secondary aims were to examine the clinical and biochemical correlates of hepatic steatosis and NAFLD and determine the diagnostic accuracy of liver enzymes in predicting hepatic steatosis in this population. The results may have practical consequences in determining the optimal mode of screening these patients within clinical practice.

### **3.1.4 Prevalence and markers of advanced liver disease in Type 2 diabetes**

Type 2 diabetes has been identified as a risk factor for the progression of NAFLD to fibrosis and cirrhosis. Despite this there are few data that have assessed the prevalence of more advanced liver disease and its sequelae in populations of people with Type 2 diabetes.

In this section, the prevalence of hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC) were assessed. The invasive nature of liver biopsy makes its use unethical in large scale studies of essentially healthy volunteers. Therefore non-invasive markers were used in this study – HA and clinical risk scores as indicators of hepatic fibrosis, ultrasound to diagnose cirrhosis and (in combination with alpha-fetoprotein (AFP)) HCC, and platelet count/spleen diameter ratio as a surrogate marker of portal hypertension. The relationship of liver enzymes with markers of advanced liver disease was also examined. In the clinical setting, accurate identification of patients with more advanced liver disease allows screening for complications and, if appropriate, consideration for clinical trials of disease modifying agents. The results of this study were anticipated to guide means of detection of such patients and subsequent service provision.

### **3.1.5 Relationship of hepatic steatosis and NAFLD with clot formation and lysis dynamics, and pro-thrombotic mediators**

NAFLD has been associated with a higher prevalence of cardiovascular disease than is present in the general population. Emerging risk factors for cardiovascular disease in this context include a systemic pro-thrombotic tendency, and one area of interest is inter-individual differences in formation and structure of the fibrin-rich clot and its lysis.

The aim of this study was to examine the relationship between hepatic steatosis and NAFLD and measures of clot formation, density and lysis. It was hypothesised that steatosis and NAFLD would be associated with a prothrombotic tendency – specifically greater clot density, longer clot lysis times and higher levels of pro-thrombotic and inflammatory mediators. By studying this pathway it was hoped to provide further insight into mediators of cardiovascular risk in people with NAFLD, and one might anticipate that this might provide treatment targets in the future.

## Chapter 4

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### Methods

#### 4.1 The Edinburgh Type 2 Diabetes Study

All work described in subsequent chapters was performed within the Edinburgh Type 2 Diabetes Study (ET2DS) cohort. The background to this study, along with the ascertainment and recruitment of study participants will be described in detail in the present chapter.

##### 4.1.1 Background

The ET2DS was initially conceived as a population-based prospective cohort study, designed to examine cognitive function and factors associated with its decline in a population of older people with Type 2 diabetes. At baseline, in a year-long period spanning 2006 – 2007, over 1000 participants attended the dedicated research clinic and underwent a variety of assessments, with a focus on detailed cognitive testing and assessment of modifiable risk factors including microvascular disease, hormones of the hypothalamic-pituitary-adrenal axis and inflammatory mediators. The recognition of the contribution that this well-characterised Type 2 diabetic cohort could make to the understanding of the epidemiology of hepatic steatosis and NAFLD gave rise to the “liver study” that is the focus of this thesis.

##### 4.1.2 Participant selection and recruitment

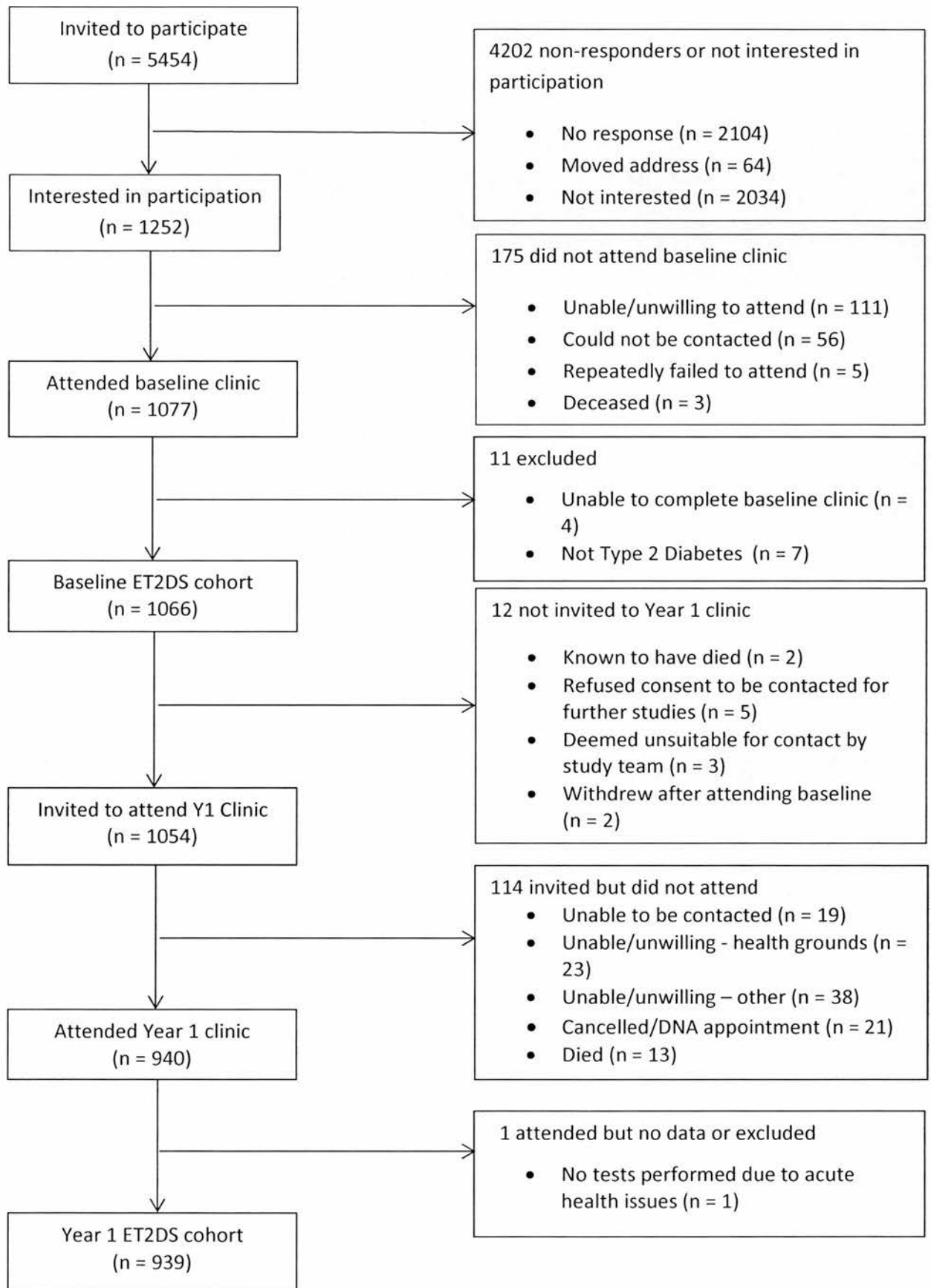
Study participants were selected from over 20000 patients recorded as having Type 2 diabetes on the Lothian Diabetes Register (LDR). The LDR is a computerised database, established in 2001, that contains details of all people with ascertained diabetes living in the Lothian region of Scotland. Thus the study population contained both patients looked after within primary care and those seen within a hospital clinic. Inclusion criteria were an age between 60 and 74 years, and a diagnosis of Type 2 diabetes. Exclusion criteria were: (1) non-English speakers (as fluent English was required for the cognitive testing); (2) visual

acuity worse than 6/36 for distance vision or unable to read large print text (as moderate vision was required for cognitive testing); (3) unwilling or unable to give informed consent; (4) physically unable to complete the clinical or cognitive examination.

People with Type 2 diabetes aged between 60 and 74 years on 1<sup>st</sup> August 2006 were selected by gender and 5-year age bands from computer-randomised lists of eligible subjects extracted from the LDR. Between 20<sup>th</sup> June 2006 and 1<sup>st</sup> June 2007, 5454 invitations to participate in the study were mailed by the custodians of the LDR. Of these, no response was received from 2104, and a further 64 were returned as being unknown at the address recorded on the LDR. Of the 3286 individuals who replied, 1252 were interested in participating in the study. Another randomly-selected subject, from the same sex and five-year age band, replaced subjects that had not replied or were disinclined to participate. Significant efforts were made to ensure that all who agreed to participate were actually assessed, including multiple contacts by the ET2DS team, payment of travel expenses and provision of transport if required, a choice of clinic dates and reminder telephone calls on the day preceding the appointment. A total of 1077 participants attended, and of the remainder 56 could not be contacted, 111 were unwilling or unable to attend, 5 repeatedly failed to attend and 3 had died. Of the 1077 participants, 4 were excluded as they were unable to complete the physical or cognitive examinations and 7 were excluded as they did not fulfil the criteria for a diagnosis of Type 2 diabetes as outlined below. The final baseline study population numbered 1066 participants, as summarised in Figure 4.1.

One year later, all original participants who were alive and had consented to be contacted about additional studies were invited to return to the Year 1 clinic, whose focus was on hepatic assessment. Of the 1066 participants in the baseline, 1054 were invited to re-attend. Of the remainder, 2 were known to have died, 5 had refused consent to be contacted about further studies, 3 were deemed unsuitable for contact by study team and 2 had withdrawn from any further contact after the baseline examination. A total of 940 subjects attended the Year 1 clinic. Reasons for not attending were: unable to contact subject (n = 19); unable/unwilling to attend on health grounds (n = 23); unable/unwilling to attend for other reasons (n = 38); cancelled/did not attend appointment (n = 21); and death (n = 13). One of

Figure 4.1  
Participants in the Edinburgh Type 2 Diabetes Study





the participants was unable to complete the examination and the study population for the Year 1 liver study therefore numbered 939 participants (Figure 4.1)

#### **4.1.3 Confirmation of Type 2 diabetes diagnosis**

The study went to considerable lengths to ensure that that diagnosis of Type 2 diabetes, as entered on the LDR by the patient's primary or secondary care physician, was correct. A diagnosis of diabetes was accepted in any individual treated with oral antidiabetic agents and/or insulin, and in any individual treated with dietary modification alone whose HbA1c was  $> 6.5\%$ . The clinical records of all subjects treated with dietary modification alone and with an HbA1c  $\leq 6.5\%$  at the research clinic were reviewed by a consultant diabetologist (Dr Mark Strachan) to ensure that the diagnosis of diabetes was accurate. With regard to the classification of 'Type 2' diabetes, the clinical records of individuals who either: (1) started on insulin within one year of diagnosis of diabetes, (2) reported evidence of pancreatic surgery or disease at the research clinic or (3) were treated with insulin and were aged  $< 35$  years at diagnosis were also reviewed. Such individuals were considered to be at greatest risk of mis-classification. Any subject in whom it was not possible to confirm a clinical diagnosis of type 2 diabetes by review of hospital or general practitioner records was excluded as has been outlined above.

#### **4.1.4 Power calculation and sample size**

The original power calculation was based upon requirements for baseline and follow-up testing of the cognitive function variables(281). With a sample size of 1000 it was calculated that there would be a 90% power at the two-sided 5% level of significance to detect a Pearson correlation co-efficient of  $\geq 0.10$  between the continuous outcome measures and predictor variables, and with expected drop-outs and deaths a sample size of 800 would have 90% power to detect a correlation coefficient of  $\geq 0.12$  between risk factors and outcome measures. It was also estimated that, with a population of 800, it would be possible to detect any risk factor that contributed 1% or more to the variance in outcome.

#### **4.1.5 Ethical permission and consent**

Ethical permission for both the baseline and liver studies was granted by the Lothian Medical Research Ethics Committee. The Lothian Diabetes Services Advisory Group and the Caldicott Guardian for NHS Lothian gave permission for the use of the Lothian Diabetes Register in patient selection. Participants gave written informed consent at both baseline and liver clinic visits.

## 4.2 Data collection

### 4.2.1 Baseline Clinic

Subjects attended the baseline clinic after an overnight fast for an assessment that included physical examination and venepuncture. Examination was carried out by one of six trained research nurses and measurement technicians, using pre-specified standard operating procedures. Subjects also submitted a completed questionnaire including information on demographic characteristics, diabetes history and treatment, medication use, alcohol intake and smoking history. A full summary of data points collected at baseline is summarised in a published study protocol(281). Only these that are directly applicable to the work presented here will be considered further at present.

#### *Body mass index*

Standing height was measured to the nearest millimetre using a wall-mounted vertical rule (without shoes). Weight was assessed to the nearest 0.1 kg without outdoor clothing or shoes using SECA 761 electronic weighing scales. BMI was defined in the usual manner as weight (kg) divided by the square of the height (m).

#### *Waist circumference*

Waist circumference was measured during exhalation at the level midway between the lower rib margin and the iliac crest (pre-marked at the mid-axillary line on each side of the subject) with the subject standing with feet 30 cm apart and hands by their sides. Measurement was made with a non-expandable tape measure. The average of two readings taken to the nearest 0.5 cm was taken as the final measurement.

#### *Venepuncture*

Venous blood samples were taken at baseline after an overnight fast. Fibrinogen levels were assessed on plasma samples using the automated Clauss assay (MDA-180 coagulometer, Organon Teknika). CRP was assayed using a high-sensitivity immunonephelometric assay. TNF $\alpha$  and IL-6 levels were determined using high-sensitivity ELISA kits (R&D Systems,

Oxon, UK). Hyaluronic acid (HA) was measured using a radiometric assay (Pharmacia, Uppsala, Sweden).

### *Record linkage*

Details of discharge diagnoses following admission to any non-psychiatric, non-obstetric ward in a Scottish hospital, between 1981 and September 2007, were collected on all participants at baseline, using record linkage from the Information and Services Division of NHS Scotland.

### **4.2.2 Year 1 (“liver”) clinic**

Subjects attended the Year 1 clinic after a four-hour fast. The main procedure performed was ultrasound of abdomen and the details of this are described in Chapters 5 and 6. In addition subjects had BP and venepuncture performed as outlined below. Questionnaires were submitted with details of alcohol consumption (as described in Chapter 6), medication use and history of liver or joint disease.

### *Blood pressure*

Systolic and diastolic blood pressures were measured twice after subjects had been supine for at least 25 minutes, and the mean of each used. Either an aneroid 6 inch dial, desk standing sphygmomanometer or a mercurial sphygmomanometer was used. All measurements were taken from the left arm unless this was undesirable or impossible.

### *Venepuncture*

Venous blood samples were obtained after a 4-hour fast. Bilirubin, ALT, AST, AlkP, GGT, AFP triglycerides, total and HDL cholesterol were measured on the Vitros Fusion chemistry system (Ortho Clinical Diagnostics, Bucks, United Kingdom). Ferritin was measured by immunoassay on the ADVIA Centaur (Siemens Healthcare Diagnostics Inc., Illinois, USA). Hepatitis B surface antigen and hepatitis C antibody index were measured on the Abbott Architect immunochemistry system (Abbott Laboratories, Illinois, USA). ANA, anti-smooth

muscle antibody and anti-mitochondrial antibody were measured by immunofluorescence using the Beeline slide analyser (HTZ Ltd, London, UK). PAI-1 and C3 levels were determined using ELISA kits (Invitrogen, Paisley, UK, and GenWay Biotech, San Diego, USA respectively).

Collection and analysis of samples for clot lysis dynamics is described in Chapter 8.

### 4.3 Representativeness Data

Non-identifiable data were gathered from the LDR on those who were invited to attend but did not. Baseline study participants (n=1066) were shown to be representative, in terms of age, HbA1c, duration of diabetes, insulin treatment and total cholesterol, of all those randomly selected to participate (n=5454) and therefore of the target population of older men and women with type 2 diabetes living in the general population(282). There was a significant difference in the percentage of men in the study compared to the percentage invited, and this was due to the methodology of the study which demanded similar numbers of men and women in each age range. Information on the spread of participants looked after within primary and secondary care was not available, but the other parameters examined suggested that the population was likely to be representative. Representativeness data are shown below in Table 4.1.

Table 4.1

Representativeness data from the ET2DS cohort(282).

<b>n</b>	1066	4386
Age (years)	67.9 ± 4.2	67.9 ± 4.4
Gender (% male)	51.3	41.9*
Duration diabetes (% ≥ 5 years)	51.6	51.3
HbA1c (%)	7.4 ± 1.1	7.4 ± 1.4
Insulin user (%)	17.4	16.1
sBP (mm Hg)	133.3 ± 16.4	137.2 ± 18.2*
Total cholesterol (mmol/l)	4.3 ± 0.9	4.2 ± 1.0

\*p < 0.01 on student's t-test or chi-squared analysis.

Representativeness of the Year 1 cohort is considered in Chapter 6.

### **Analysis 1: Use of Ultrasound to Diagnose Hepatic Steatosis**

#### **5.1 Introduction**

Hepatic steatosis and NAFLD are commonly found within the general population and, as outlined in detail in Chapter 2, there is evidence to support a higher prevalence of these conditions within populations of people with Type 2 diabetes(18;170;171). It is important to accurately detect this condition as it may result in considerable morbidity and mortality in this patient group(99;182;209-211), and can independently predict the risk of cardiovascular events(223;271). Its identification can be used to initiate screening and target treatment for more advanced liver disease.

##### **5.1.1 Evidence for the diagnostic utility of ultrasound in the detection of hepatic steatosis**

Ultrasound imaging is the predominant imaging modality in the diagnosis of hepatic steatosis, both clinically and in large-scale studies. Although several grading systems have been proposed for the assessment of hepatic steatosis using ultrasound, no consensus has been achieved(16;80-83). Detection of hepatic fat is based on well-established characteristics including an echogenic parenchyma (especially in relation to the right kidney), posterior attenuation and areas of focal fatty sparing(16;82-85). Compared with liver biopsy it has been reported to have a sensitivity of 53-100% and specificity of 77-100% in the diagnosis of hepatic steatosis(75;76;80;82;84;86); advantages over biopsy include safety, ease of acquisition and ability to assess the whole liver. The variability in diagnostic accuracy of ultrasound is likely to be due to a number of factors as described in Chapter 1, including differing levels of obesity between populations and evolving scanning technology.

Although a degree of subjectivity is recognised when diagnosing hepatic steatosis by ultrasound examination, few studies have examined intra- and inter-observer variability in

making this diagnosis. One study of 25 patients with biopsy-proven NAFLD (less than a quarter of whom had diabetes) reported that the inter-observer correlation between two radiologists in determining severity of steatosis was 0.40 (representing fair agreement), and the intra-observer correlation was 0.63 (suggesting more substantial agreement)(80). A different study - of 168 patients - who had undergone abdominal ultrasonography for a variety of abdominal disorders, demonstrated inter-observer agreement between three radiologists on the presence and severity of hepatic steatosis, of 0.40-0.51 and intra-observer agreement of 0.58 (moderate agreement)(88). In both studies, the assessment of the severity of steatosis was based on the presence and severity of increased echogenicity of the hepatic parenchyma and attenuation of the ultrasound beam. Neither study was carried out in people with Type 2 diabetes, in whom one might conjecture rates of obesity would be higher than in the general population.

A valid inference from the above data is that studies employing ultrasound imaging in the diagnosis of hepatic steatosis should validate their ultrasound measure. This, however, has not been commonly carried out.

### **5.1.2 MRS in the measurement of hepatic steatosis**

In comparison to USS, MRS, the non-invasive gold standard for measurement of hepatic fat, is expensive but has a high correlation with liver fat concentration on biopsy, of the order of 0.7 - 0.9(70;71). Different “normal” values for hepatic FF on magnetic resonance imaging have been reported previously(63;73;74). The Dallas Heart Study used MRS to determine hepatic FF in a population of 345 participants who had normal BMI, glucose tolerance and LFTs and non-excessive alcohol use (i.e. a very low risk for developing hepatic steatosis). In this group the 95<sup>th</sup> percentile of hepatic triglyceride content (expressed as grams triglyceride per 100 grams wet liver tissue) was 5.5%, corresponding to an MR FF of 6.1%(70), and this was taken as the upper limit of normal(63). In an earlier smaller study, 28 healthy volunteers underwent MR scanning by a modified Dixon gradient echo technique. All had a MR hepatic FF under 9% and this was later considered as being the upper limit of normal(73;74). In the same study seven subjects with cystic fibrosis underwent both MRI scanning and liver biopsy: two subjects with no steatosis on biopsy had a FF on MRI of < 9%; five subjects with mild to severe steatosis had a FF of > 9%.



No study has compared qualitative ultrasound gradings with FF on MRS. This is particularly relevant as there are theoretical advantages of MRS over biopsy in terms of the higher proportion of liver sampled and possible reduction in inter-observer variability.

### **5.1.3 Study aims**

The aims of the present study were to compare ultrasound gradings of hepatic steatosis, performed by three graders, with hepatic FF on MRS and also to examine inter- and intra-observer variability in these ultrasound gradings.

## 5.2 Methods

### 5.2.1 Ultrasound assessment

The selection of subjects for the Edinburgh Type 2 Diabetes Study has been described in detail in Chapter 4. A total of 939 participants attended for liver assessment by liver ultrasound examination (USS<sub>l</sub>) at a specially set up research clinic. All ultrasound examinations were performed after a four-hour fast by a single ultrasonographer (Grader 1), using a Sonoline Elegra Ultrasound Imaging System (Sieman's Medical Systems Inc, Washington, USA), software version 6, with a 3.5 MHz transducer. The appropriate pre-set ("medium" or "large" abdomen) was chosen for each subject according to the ultrasonographer's judgement – these pre-sets remained constant throughout the study. Participants were scanned raised at an angle of 30-40° onto their left side. Right lobe images were subcostal or intercostal depending on the body habitus of the participant; left lobe images were obtained in the midline of the abdomen. The following images were obtained: longitudinal images of the right lobe and caudate lobe with inferior vena cava, right lobe with the portal vein, right lobe in the mid-clavicular line, right lobe of liver with kidney (at least two images) for comparison, right lobe close to the diaphragm and left lobe in its midline; transverse images of the right lobe at the level of the hepatic veins entering the inferior vena cave and at the level of the main portal vein, the right lobe close to the diaphragm, the right lobe at the level of the right portal vein and /or right hepatic vein, the right lobe of liver with kidney for comparison and the left lobe at the level of the left hepatic vein and/or left portal vein. The liver was graded for evidence of hepatic steatosis using accepted criteria including a bright hepatic echo pattern (compared with the right kidney), increased attenuation of the echo beam (impaired visualisation of the diaphragm or intrahepatic vessels) and the presence of focal fatty sparing(16;80-83). Participants were given an overall liver grading based on a subjective measurement of the severity of steatosis: *normal*; *indeterminate* (i.e. possible slight increase in echogenicity or slightly impaired visualisation of the diaphragm or intrahepatic vessels, or difficulty in grading as a result of a diseased or absent right kidney); *mild steatosis* (i.e. definite increase in echogenicity and/or definite impaired visualisation of the intrahepatic vessels and diaphragm, no or little evidence of focal fatty sparing); *severe steatosis* (i.e. marked increase in echogenicity and/or poor or no visualisation of the diaphragm and intrahepatic vessels, with or without focal fatty sparing).

### **5.2.2 MRS Subgroup: participant selection**

A subgroup of participants from the cohort described above were selected to have MRS. 20 participants were selected at random into each of three groups on the basis of their USS<sub>1</sub> grading: normal (with normal biochemical tests of liver function); indeterminate or mild steatosis (a pre-determined merging of these two gradings into one group); and severe steatosis. In addition, those with steatosis were selected following exclusion of a secondary cause (excess alcohol intake, hepatotoxic medication use, viral hepatitis or autoimmune hepatitis) and therefore had NAFLD. Other exclusion criteria were maximal diameter over 60 cm (in order to fit the dimensions of the scanner) and contraindications to magnetic resonance imaging. One participant who did not tolerate the procedure was replaced by another participant who had the same grading classification. MRS measurements were unsuccessful in two participants and therefore 58 participants who attended for imaging had successful FF measurements.

### **5.2.3 MRS subgroup: study protocol**

At the MRS visit, participants underwent a repeat ultrasound scan (USS<sub>2</sub>), using the above protocol, 2 hours prior to MRS. This measurement was made to ensure that the ultrasound grading reflected the presence or absence of steatosis in the liver at the time of MRS.

The MRS examination was performed using a 1.5T HDX scanner (GE Healthcare, Amersham, UK) and consisted of a PRESS-localised volume of interest (VOI) of dimensions 30 × 30 × 30 mm, placed away from vascular structures. The scanner's body coil was used for transmission and reception. A repetition time (TR) of 5 s allowed substantial T1 relaxation of the signals, and a short (40 ms) echo time (TE) was used to minimise T2 decay. Automatic shimming achieved a water linewidth of typically 11 Hz. Sixteen acquisitions were averaged. Water-suppressed spectra (64 acquisitions) were also collected but are not reported here. The MRS data files were transferred from the scanner to a computer workstation for analysis. Quantification consisted of normalisation for scanner transmit and receive gains and actual VOI volume, followed by modelling of the water and fat peaks using the MRUI package ([www.mrui.uab.es](http://www.mrui.uab.es)). FF was defined as the ratio of the area under the fat peak to the combined areas under the water and fat peaks.

#### **5.2.4 Intra- and Inter-observer Variability**

Sixty participants from the MRS subgroup had USS<sub>2</sub>, and 71 participants from the initial cohort had USS<sub>1</sub>, graded by three independent graders using the four-point grading system outlined above (normal, indeterminate, mild steatosis and severe steatosis). The 71 participants from the initial cohort represented all who were scanned during a particular calendar month. The three graders comprised the sonographer who performed the scans (grader 1, 15 years scanning experience), a consultant radiologist (grader 2, 12 years scanning experience) and a medical trainee in radiology (grader 3, 2.5 years scanning experience). Grader 1 scored images at the time of acquisition; still images from the scans were recorded and saved for examination by graders 2 and 3. The three graders were blinded to each other's gradings and to the MRS result. All graders were unaware of the clinical and laboratory results of the participants.

After an interval of at least two months, each of the three graders re-graded the still images from the 60 USS<sub>2</sub> scans, in a blinded fashion, to allow assessment of intra-observer variability. Inter-observer variability in these re-gradings was also examined, as this was the only instance when all three graders were grading from still images.

#### **5.2.5 Statistical Analysis**

Median and inter-quartile ranges of FF on MRS were calculated in each group according to ultrasound gradings. The Kruskal Wallis test was used to compare FF between all three groups, and the Mann-Whitney test was used for post-hoc comparisons between pairs of groups. Sensitivity and specificity of the USS<sub>2</sub> gradings were determined with reference to MRS data, using two separate cut-offs of FF to define "normality"– 9% and 6.1%(63;70;74). The results of sensitivity and specificity analysis for Grader 1 were later adjusted to take into account the proportion of subjects in the entire cohort receiving each grading. Inter- and intra-observer variability were analysed using the marginal homogeneity test. Data were analysed using SPSS version 14.0.

## 5.3 Results

### 5.3.1 MRS Subgroup: comparison of hepatic FF between participants grouped according to ultrasound grading

All 58 subjects with spectroscopy measurements had USS<sub>2</sub> graded by three graders; the number of participants put into each category for each grader is noted below in Table 5.1.

For Grader 1, median MRS FF was 4.2% (interquartile (IQ) range 1.2 – 5.7%) in the group graded normal, 4.1% (IQ range 3.1 – 8.5%) in the group graded indeterminate/mildly steatotic and 19.4% (IQ range 12.9 – 27.5%) in the group graded severely steatotic (Figure 5.1). As shown in Figure 5.1, there were two outliers in the indeterminate/mildly steatotic group, both with a FF on MRS of over 30%. There was a significant difference in median FF between the three groups ( $p < 0.001$ ). The group with severe steatosis had significantly higher FF than either the normal ( $p < 0.001$ ) or the indeterminate/mild steatosis groups ( $p < 0.001$ ). There was no significant difference in FF between the normal and indeterminate/mild steatosis groups. Results were similar for the gradings of the other two graders (Table 5.1).

Table 5.1

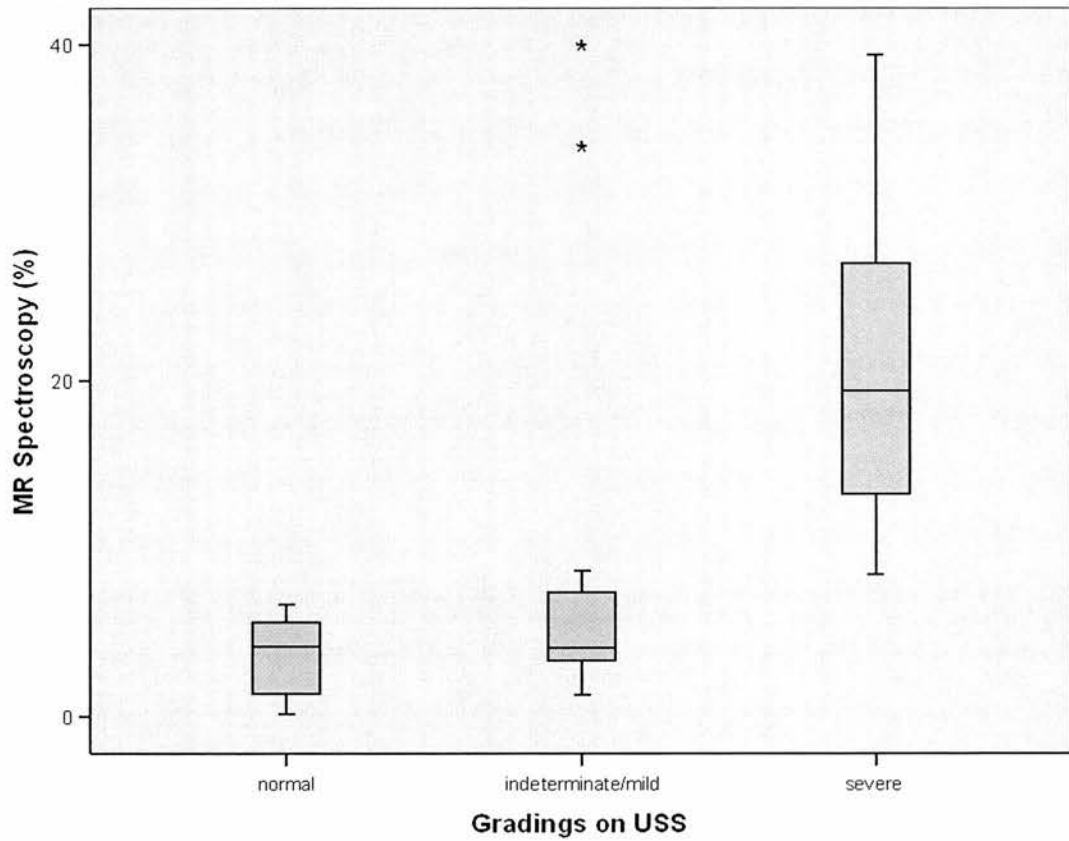
Median (interquartile range) FF on MRS for each of three groups of gradings on USS (normal, indeterminate/mildly steatotic, severely steatotic) as performed by three graders. The number (N) of participants placed in each group by each grader is also noted.

	Normal	Indeterminate/mild	Severe
Grader 1	4.2 (1.2 – 5.7) N = 17	4.1 (3.1 – 8.5) N = 19	19.4 (12.9 – 27.5)* N = 22
Grader 2	4.2 (1.3 – 5.7) N = 16	4.1 (2.6 – 7.9) N = 20	19.4 (12.9 – 27.5)* N = 22
Grader 3	4.2 (1.8 – 6.3) N = 26	6.0 (3.0 – 12.5) N = 14	20.1 (12.9 – 28.4)* N = 18

\* significant difference in FF between the normal, indeterminate/mild and severe groups ( $p < 0.001$ )

Figure 5.1

Boxplot showing median percentage fat on MR spectroscopy in each of three groups of ultrasound gradings by Grader 1 (normal, indeterminate/mild steatosis, severe steatosis). Upper and lower ends of rectangles correspond to 75<sup>th</sup> and 25<sup>th</sup> centiles respectively. Whiskers represent maximum and minimum values. Outliers in the indeterminate/mild steatosis group are marked by \*. The group with severe steatosis had a significantly higher median FF than those with normal or indeterminate/mild steatosis ( $p < 0.001$ ).



### 5.3.2 MRS subgroup: diagnostic utility of ultrasound gradings compared with MRS FF

For Grader 1, all 17 subjects in the group who were graded as normal on USS<sub>2</sub> had a FF less than 9%, and 14 of these subjects had a FF less than 6.1%. In the indeterminate/ mildly steatotic ultrasound group, 16 subjects had a FF less than 9% and, of these subjects, 10 had a FF under 6.1%. In the severely steatotic group, all but one participant had a FF of greater than 9%, with the remaining subject having a FF of 8.45%. Results for the other two graders were similar and are shown in Table 5.2.

Table 5.2

Comparison of ultrasound gradings with proposed cut-offs of "normality" of FF on MRS – < 6.1% and 9%(63;73)

Grading →		Normal			Indet/mild			Severe		
Grader →		1	2	3	1	2	3	1	2	3
Total no. participants →		17	16	26	19	20	14	22	22	18
MRS cut-off 9%	< 9%	17	16	23	16	17	10	1	2	2
	≥ 9%	0	0	3	3	3	4	21	20	16
MRS cut-off 6.1%	< 6.1%	14	14	19	13	13	7	0	0	1
	≥ 6.1%	3	2	7	6	7	7	22	22	18

Sensitivity and specificity were calculated with both MRS FF cut-offs and results, with 95% confidence intervals, are presented in Table 5.3. For Grader 1 using a liver FF of  $\geq 9\%$  on MRS to denote hepatic steatosis, sensitivity of ultrasound in detecting indeterminate/mild/severe steatosis was 100% and specificity was 50.0%. If those graded indeterminate/mild steatosis were instead grouped with those graded normal (in view of the similar median MRS FF in these groups) sensitivity was 87.5% and specificity 97.1%. When a liver FF of  $\geq 6.1\%$  was used to define hepatic steatosis, sensitivity of ultrasound in detecting indeterminate/mild/severe steatosis was 90.3% and specificity was 51.9%. When those with indeterminate/mild steatosis were grouped with those graded normal, sensitivity was 71.0% and specificity 100%. Results were extremely similar for grader 2. For grader 3, sensitivity in detecting indeterminate/mild/severe steatosis was lower (87.0% and 78.1% for the 9% and 6.1% cut-offs respectively) but specificity was higher (65.7% and 57.6% for the 9% and 6.1% cut-offs respectively).

Since sensitivity and specificity of a test can be affected by the prevalence of the underlying condition (and in our study, subjects were selected according to presence of the condition on USS, thereby pre-determining the prevalence), the results of Grader 1 were adjusted to take account of the proportion of subjects in the entire cohort of 939 subjects who received each grading (24% normal, 19% indeterminate/mild steatosis, 57% severe steatosis). The results were not substantially different from the unadjusted data (Table 5.3).



Table 5.3

Sensitivity and specificity of ultrasound in identifying presence of hepatic steatosis, using two cut-offs of hepatic FF on MRS (<9% and <6.1%). Data are shown for the grouping of the indeterminate/mild steatosis group with either the normal or the severe steatosis groups. Both unadjusted values and those that have been adjusted to take account of the proportion of each grading in the entire study cohort are presented. Ultrasound gradings are from Grader 1. Data are presented as % (95% confidence interval).

	Sensitivity (unadjusted)	Specificity (unadjusted)	Sensitivity (adjusted)	Specificity (adjusted)
9% cut-off; Normal vs indet/mild/severe steatosis	100 (82.8 - 100)	50.0 (32.8 - 67.2)	100 (87.0 - 100)	56.0 (35.2 - 75.0)
9% cut-off, Normal/indet/mild steatosis vs severe steatosis	87.5 (66.5 - 96.7)	97.1 (82.9 - 99.8)	93.9 (78.4 - 99.0)	92.0 (72.4 - 98.6)
6.1% cut-off, Normal vs indet/mild/severe steatosis	90.3 (73.1 - 97.5)	51.9 (32.4 - 70.8)	94.7 (80.9 - 99.1)	60.0 (36.4 - 80.0)
6.1% cut-off, Normal/indet/mild steatosis vs severe steatosis	71.0 (51.8 - 85.1)	100 (84.5 - 100)	86.8 (71.1 - 95.1)	100 (80.0 - 100)

### 5.3.3 Inter- and intra-observer variability

Inter-observer variability was determined in a group of 131 participants, comprising the 60 subjects in the MRS subgroup and scans from a further 71 subjects from the original cohort. Calculation of inter-observer agreement between the three graders revealed exact consensus in 51% (67/131 given the same grade). Inter-observer agreement within one grade was seen in 79% of cases (104/131). Inter-observer variation of 2 grades was seen in 17% (22/131) and variation of 3 grades was seen in 4% (5/131). On pairwise analysis, there was no significant variation in the gradings of Graders 1 and 2 (68 % exact consensus), or between those of Graders 2 and 3 (65% exact consensus). When the gradings of Graders 1 and 3 were compared, however, there was found to be significant variation (disparity in 41% of results,  $p = 0.024$ ).

When the 71 subjects from the original cohort were examined separately, there was no significant variation between gradings from different graders. In contrast, there was significant variation between the gradings of the 60 participants in the MRS subgroup between Graders 1 and 3 ( $p = 0.005$ ), and Graders 2 and 3 ( $p = 0.011$ ).

For the 60 participants in MRS group, inter-observer analysis was repeated using the three graders' second grading, so that all three graders were using still images for grading purposes. Exact consensus was achieved in 57% (34/60 given the same grade). Inter-observer agreement within one grade was seen in 68% (41/60). Inter-observer variation of 2 grades was seen in 25% (15/60) and variation of 3 grades was seen in 7% (4/60). On pairwise analysis, variability between gradings was similar to that seen for the cohort as a whole (71% exact consensus between Graders 1 and 2; 65% exact consensus between Graders 2 and 3; and 60% exact consensus between Graders 1 and 3).

Intra-observer variation was minimal for Graders 1 and 2 (87% and 77% exact concordance between the 1<sup>st</sup> and 2<sup>nd</sup> readings respectively), but statistically significant for Grader 3, with 38% disparity between readings,  $p = 0.039$ ).

## **5.4 Discussion**

To my knowledge this is the first study to examine both the relationship between ultrasound gradings of steatosis and MRS, and the inter- and intra-observer agreement in ultrasound grading. The present study was broad in its scope, including both participants who were likely to have a normal liver and those considered to have varying degrees of hepatic steatosis, mainly NAFLD. In addition, the use of ultrasound has been examined in diagnosing hepatic steatosis in people with Type 2 diabetes, a population who are at high risk of developing hepatic steatosis, and in whom screening for the condition may be considered.

### **5.4.1 MRS as gold standard: comparison of methodologies**

The current study has relied on MRS as the non-invasive gold standard (against which our ultrasound measure has been validated) and has utilised data from previous studies, in particular the Dallas Heart Study(63), in order to define “normal” values for hepatic triglyceride content. This necessitates justification of the MRS protocol used in the present study, and comparison between this and the protocol used in the Dallas Heart Study. The main aspects of scanning methodology are listed in Table 5.4.

Table 5.4

Comparison of MRS scanning methodologies in the ET2DS and the Dallas Heart Study.

	ET2DS	Dallas Heart Study
Magnetic field strength	1.5T	1.5T
VOI	3x3x3 cm	3x3x3 cm
TR	5 seconds	3 seconds
TE	40 milliseconds	25 milliseconds
Measurement reported	Fat fraction – ratio of methylene to combined methylene and water signals	Hepatic triglyceride concentration – ratio of methylene to combined methylene and water signals, expressed as weight percent (g triglyceride per 100 g wet liver tissue)

T, Tesla; VOI, volume of interest; TR, repetition time; TE, echo time

In the ET2DS, the magnetic field strength capacity of the scanner available was 1.5T and this has been similar in other studies including the Dallas Heart Study(63;283;284). The use of a higher field strength (e.g. 3T) would have been theoretically associated with the advantage of a higher signal-to-noise ratio(285), but this higher signal-to-noise ratio is not always achievable, and in the case of fat and water spectroscopy does not represent a significant benefit. Longer repetition times (> 2 seconds) and shorter echo times have been shown to improve spectral quality: the longer repetition time in the ET2DS (compared to the Dallas Heart Study and other MRS studies) meant that correction for relaxation effects was unnecessary; the echo times used in the ET2DS and Dallas Heart Study were comparable, and other studies have quoted echo times of 10 – 135 milliseconds(283;284). Although the final data reported in the two studies were different (fat fraction in the ET2DS; hepatic triglyceride concentration in the Dallas Heart Study) both studies initially calculated a ratio of fat to combined fat and water signals, with the Dallas Heart study then going on to express this in terms of weight percent of wet liver tissue. It is therefore valid to use the algebraic equations adopted in this study(70) to transform their cut-point for normal hepatic

triglyceride content back to hepatic fat fraction for comparison with the data obtained in the ET2DS.

#### **5.4.2 Comparison of USS gradings with MRS**

When compared to MRS, a normal ultrasound grading was an excellent predictor of a normal hepatic FF, and a severe steatosis grading was an excellent predictor of the presence of hepatic steatosis. The grading of a mildly steatotic liver was less secure, and in particular considerable overlap in hepatic FF was evident compared with those who had a normal liver on ultrasound. It is therefore reasonable to suppose that those graded indeterminate/mild steatosis had subtle changes on ultrasound that were within the limits of normality. An alternative explanation is that the relatively bright liver seen in the “mild steatosis” group is indicative of another pathology, such as inflammation or fibrosis. It is notable that two subjects with very high FF were graded indeterminate/mild steatosis and, in grouping these participants with the normal group, the diagnosis of steatosis would have been missed. In clinical practice, a grading of indeterminate/mild steatosis should be interpreted in conjunction with clinical and biochemical factors, and it may be appropriate to repeat the ultrasound scan after an interval of time. The performance of the three graders was similar when gradings were compared to MRS. It should be emphasised that USS images were obtained within hours of MRS measurements to ensure that they were truly comparable, as hepatic steatosis can vary over time.

Individuals were pre-selected into the MRS subgroup based on their USS<sub>1</sub> gradings, with the intention of having broadly equal numbers of participants in each of three groups. It was acknowledged that the proportion in each group was different to the proportion of each grade in the entire cohort, as graded by Grader 1. Adjusted figures were therefore provided for comparison, with the assumption that if the number of subjects in each USS grading group had been selected according to overall proportion in the total cohort, then the proportion of subjects in each group testing below and above the cut-offs of MRS FF would have stayed the same

No accepted definition of “normal” FF on MRS currently exists. Biopsy data from normal livers and MRS FF have rarely been compared. Furthermore, studies looking at “healthy”

volunteers may be skewed by the presence of overweight individuals or those who drink alcohol in amounts greater than 14 units/week – both of which are known to predispose to hepatic steatosis. It seems likely that the proposed figure of 6.1% (corresponding to 5.5% hepatic triglyceride content, if expressed as grams triglyceride per 100 grams wet liver tissue) for the upper limit of normal is the most accurate available, as this was calculated using a population from which those with risk factors for steatosis, such as BMI > 25 kg/m<sup>2</sup>, glucose intolerance and excessive alcohol use had been excluded(63).

It is acknowledged that MRS gives information regarding FF within a portion of the liver rather than in the liver as a whole, and that it was theoretically possible that participants with focal fatty change could be misclassified as a result. In this study, however, the volume of interest for MRS was fairly large in order to minimise any effects of local heterogeneity. Indeed, examination of MRS data from the 15 participants with focal fatty sparing on USS revealed FF that were in keeping with their overall ultrasound grading (data not shown).

#### **5.4.3 Intra- and inter-observer variation in USS gradings**

Comparison of the gradings by three experienced graders revealed some variation, but exact concordance in a majority of cases, with most differences being small (one-point only); intra-observer concordance was high in two of three graders, but was lower for the grader with the least experience (a medical trainee in radiology). Comparison of rates of consensus between the graders did not reveal consistently better results when all three graders used still images for grading purposes (as compared with Grader 1 grading dynamic images at the time of image acquisition and Graders 2 and 3 grading from still recorded images). Of the two previous studies to examine inter- and intra-observer variability, one was a small study whose scope was limited to patients with abnormal biochemical LFTs and biopsy-proven NAFLD(80). The other was more comparable in size and scope to the present study, but results cannot be directly compared due to different statistical methods used. The present study compared gradings from graders with different training (a sonographer, a senior consultant radiologist and a medical trainee in radiology). This is reflective of clinical practice and is particularly relevant at a time when increasing numbers of ultrasound scans are being performed by experienced sonographers both clinically and within research studies.

#### **5.4.4 Limitations of the study**

Participants in the MRS sub-study were necessarily less than 60 cm in maximal diameter so excluding people with very severe obesity. It is widely acknowledged that ultrasound is more difficult in the most obese patients and it could be anticipated that accuracy (compared to MRS) and inter-/intra-observer concordance would be less in this population. It is, however, important to note that inter-observer variation in the MRS sub-group was not superior to that of the essentially randomly selected group from this general study population.

It is acknowledged that USS gradings were compared with the non-invasive gold standard (MRS) rather than the “true” gold standard (biopsy). Biopsy would have been neither feasible nor ethical in this essentially healthy study population. In addition MRS may have an advantage in terms of proportion of the liver sampled.

#### **5.4.5 Conclusions**

In conclusion, hepatic ultrasound provides a good measure of the presence of hepatic steatosis, and in particular a normal grading and severe steatosis grading were respectively excellent predictors of a normal and high MRS FF. Hepatic ultrasound has advantages over other measures of hepatic fat in its ease of use and lack of adverse side-effects and is likely to remain a mainstay of investigation, both in research and clinical situations.

### **Analysis 2: Prevalence and clinical correlates of hepatic steatosis and NAFLD in Type 2 diabetes**

#### **6.1 Introduction**

##### **6.1.1 Association of NAFLD and Type 2 diabetes**

The relationships between NAFLD and Type 2 diabetes are well established, and have been discussed extensively in Chapter 2 of this thesis. In addition to the recognised cross-sectional associations(63;92;95), NAFLD has been implicated in the pathogenesis of Type 2 diabetes(157;158;161;162) and, conversely, diabetes has been shown to be a risk factor for the progression of NAFLD to more advanced liver disease including fibrosis, cirrhosis and HCC(99;209;210). Furthermore, data suggest that some classes of anti-diabetic agents (including thiazolidinediones and incretin mimetics) may be beneficial in ameliorating NAFLD(217;286;287).

##### **6.1.2 Prevalence of NAFLD in populations of people with Type 2 diabetes**

Despite the recognised association between type 2 diabetes and NAFLD, few large-scale studies of the prevalence of NAFLD in unselected populations of people with Type 2 diabetes are available. Studies estimating the prevalence using liver ultrasound have been performed in secondary care rather than community setting and have generally examined small numbers of patients(170;171). A large study in Italian outpatients with type 2 diabetes reported that 85% had hepatic steatosis and that 70% met the criteria for NAFLD (defined in that study as hepatic steatosis without evidence of excess alcohol consumption, viral hepatitis, or causative medications)(18). This study, however, was confined to patients attending a hospital clinic and also failed to systematically identify and exclude participants with less common causes of liver disease such as autoimmune hepatitis. Furthermore, ultrasound measurements were not compared with a gold standard in the population studied, an important factor given the variable sensitivity and specificity of ultrasound assessment for hepatic steatosis as reviewed in Chapter 1.



### **6.1.3 Clinical associations of NAFLD**

The cross-sectional relationship between NAFLD and features of the metabolic syndrome, including insulin resistance, (central) obesity and dyslipidaemia has been well documented within the general population(19;20;63;93;130;143;144;149). The correlations have, however, been less frequently examined in populations of people with Type 2 diabetes. In the Italian study quoted above, there were univariate associations between NAFLD and higher age, BMI, duration of diabetes, HbA1c, triglyceride levels and blood pressure, and an inverse relationship between NAFLD and HDL cholesterol levels, but multivariate analysis was not undertaken(18). In contrast, in a smaller study within a Saudi diabetes department, no significant correlation was found between the presence of NAFLD and duration of diabetes or glycaemic control(171).

### **6.1.4 Utility of liver enzymes in screening for hepatic steatosis and NAFLD**

Screening for liver disease in Type 2 diabetes typically involves measuring circulating levels of liver enzymes that have been released from damaged hepatocytes. ALT and, to a lesser extent, GGT have been used as a surrogate markers of NAFLD(89;90), but the diagnostic utility of these measures within the general population has been questioned(63;92;93). There are, however, few analyses that have formally examined the diagnostic accuracy of ALT. In one study, using the laboratory cut-off of 39 units/l to denote an abnormal ALT level, and comparing with ultrasound findings of steatosis, the sensitivity and specificity were found to be 8.2% and 98% respectively(93). The use of a lower ALT cut-off of 19.5 units/l, derived from a ROC curve, gave sensitivity of 72% and specificity of 60%. In another study, in which the estimated prevalence of hepatic steatosis was estimated to be around 60%, the PPV of a high ALT level in predicting the presence of steatosis on ultrasonography was calculated to be 78%(94).

Laboratory reference ranges for liver enzymes rely upon historical data generated from general populations that may have included people with covert liver disease (particularly undiagnosed NAFLD). Thus, the upper part of the traditional ALT reference range may reflect values that are more typical of liver disorder than of normality. As a result, new reference ranges have been derived that are based on data from an Italian population at low risk for liver disease (no medication use and normal BMI, lipid profile and glucose); the

upper limit of normal (95<sup>th</sup> percentile) of ALT in this group was 30 units/l in men and 19 units/l in women(91). These more stringent cut-offs have not, however, been widely adopted.

### **6.1.5 Study Aim**

Given the rising incidence and prevalence of type 2 diabetes, an accurate estimate of the prevalence of NAFLD, is important to predict the number of patients who will require to be monitored for more advanced liver disease or who may benefit from future disease-modifying agents. Accurate determination of the (routinely-measured) clinical correlates of NAFLD may aid identification of those patients who should undergo screening for the condition. There is, however, justifiable concern that the current screening tool for liver disease in Type 2 diabetes (hepatic enzymes) may be inadequate for the task of identifying individuals with hepatic steatosis and NAFLD, but its accuracy has not been formally examined in the context of Type 2 diabetes.

The aim of the present study was to determine the prevalence of hepatic steatosis and NAFLD in a large randomly-selected population of older people with type 2 diabetes, using MRS to confirm ultrasound grading classifications and thorough screening for secondary causes of liver disease, and to examine their correlation with clinical and biochemical characteristics. In addition, the relationship of hepatic steatosis with standard tests of liver function, using both traditional and newly suggested reference ranges above for ALT, was to be examined with the aim of defining diagnostic accuracy of the liver enzymes.

## **6.2 Methods**

### **6.2.1 Study participants**

The recruitment and baseline examination of subjects for the Edinburgh Type 2 Diabetes Study has been described in Chapter 4. Briefly, subjects recorded as having type 2 diabetes and aged 60-74 years, were selected at random by sex and 5-year age bands in years 2006–2007 from the Lothian Diabetes Register, a comprehensive database of people with type 2 diabetes living in Lothian. The study population therefore includes both patients attending a hospital clinic and those managed solely in primary care. Study participants (n=1066) have been shown previously to be representative, in terms of age, HbA1c, duration of diabetes, insulin treatment and total cholesterol, of all those randomly selected to participate (n=5454) and therefore of the target population of older men and women with type 2 diabetes living in the general population(282). At the time of recruitment into the study, participants attended a baseline examination which included measurement of demographic and anthropometric variables (age, gender, BMI, WC), platelets and HA. One year after baseline examination, 939 participants (out of the 1054 invited) attended a Year 1 clinic for assessment of liver structure and function.

### **6.2.2 Ultrasound Examination and Comparison with MRS**

Subjects attended the year 1 research clinic after a 4-hour fast for an ultrasound examination of abdomen.

All ultrasound examinations were performed by a single ultrasonographer, who was unaware of the clinical and laboratory results of the participants, using a Sonoline Elegra Ultrasound Imaging System (Sieman's Medical Systems Inc, Washington, USA), software version 6, with a 3.5 MHz transducer. The liver was graded for markers of hepatic steatosis using established criteria(16;84;85) including a bright hepatic echo pattern (compared with the echo response of the right kidney), increased attenuation of the echo beam and the presence of focal fatty sparing. Participants were given an overall liver grading based on a subjective measurement of the severity of steatosis: Grade 0 (normal appearance of liver on ultrasound, and initially graded as a "normal ultrasound"), Grade 1 (possible slight increase in echogenicity or slightly impaired visualisation of the diaphragm or intrahepatic vessels, or

difficulty in grading as a result of a diseased or absent right kidney – initially termed an “indeterminate ultrasound”); Grade 2 (definite increase in echogenicity and/or definite impaired visualisation of the intrahepatic vessels and diaphragm, no or little evidence of focal fatty sparing, initially graded as “evidence of mild steatosis on ultrasound”); Grade 3 (marked increase in echogenicity and/or poor or no visualisation of the diaphragm and intrahepatic vessels, with or without focal fatty sparing, initially graded as “evidence of severe steatosis on ultrasound”). Evidence of hepatic cirrhosis was also sought systematically.

Comparison of the ultrasound gradings for hepatic steatosis with <sup>1</sup>H MRS, the non-invasive gold standard for quantification of hepatic fat, in a subgroup of 58 participants was described in detail in Chapter 5. In brief, 17 participants with a Grade 0 ultrasound grading, 19 with Grade 1 or 2 grading and 22 graded as Grade 3 underwent MRS. A Grade 0 grading on ultrasound was associated with a “normal” hepatic FF on MRS of < 6.1%(63;70) in 14/17 cases (and a FF of < 9%(73;74) in all cases) and was accepted as “no steatosis”. A grading of Grade 3 on ultrasound was associated with and MRS hepatic FF ≥ 6.1% in all cases and this was therefore taken as “definite steatosis”. The group with Grade 1 or 2 on ultrasound had an MRS FF of < 6.1% in 13/19 cases, and a FF of < 9% in 16/19 cases, thus displaying considerable overlap with those regarded as normal. In view of this the group with Grade 1 or 2 on ultrasound scan were considered to have a “probable normal” scan. If a liver FF of ≥6.1% on MRS was used to denote hepatic steatosis, sensitivity, specificity, positive and negative predictive values (adjusted for the portion of the whole study cohort receiving each grading) of ultrasound in detecting “definite steatosis” were 86.8%, 100%, 100% and 80.0% respectively.

### **6.2.3 Clinical Examination**

Average alcohol intake per week over the previous year, a history of alcohol excess and use of hepatotoxic medications within the previous six months were determined by questionnaire. Average alcohol intake was determined using two questions adapted from the AUDIT-C screening tool(288): “How often did you have a drink containing alcohol in the past year? Consider a “drink” to be a can or bottle of beer, a glass of wine, or one cocktail or a measure of spirits (like scotch, gin or vodka).” (A drink was considered to be the

equivalent of one unit of alcohol); and “How many drinks did you have on a typical day when you were drinking in the last year?”.

All subjects underwent venepuncture for LFTs (bilirubin, ALT, AST, AlkP and GGT), HbA1c and a lipid profile. Those participants with evidence of hepatic steatosis or abnormal blood tests of liver function had further investigations performed including serology for Hepatitis B and C, anti-nuclear antibody (ANA), anti-smooth muscle antibody, anti-mitochondrial antibody and ferritin. Brachial blood pressure (BP) was measured in all.

Information on previous diagnoses of chronic liver disease was collected from participants by questionnaire at Year 1, and supplemented using data on liver diagnoses which had been obtained at baseline via record linkage to hospital discharges at the Information and Services Division (ISD) of NHS Scotland.

#### **6.2.4 Definition of NAFLD**

NAFLD was defined as the presence of definite hepatic steatosis on ultrasound scan (i.e. Grade 3) in the absence of a secondary cause for hepatic steatosis. Secondary causes were defined as alcohol consumption  $\geq 14$  units/week(10) or participant report of current/previous alcohol excess, use of hepatotoxic medication(17) (glucocorticoids, isoniazid, methotrexate, amiodarone and tamoxifen) within the six months prior to the Year 1 clinic, positive hepatitis B or C serology, ferritin concentration  $\geq 1000$  ug/l (milder hyperferritinemia can be associated with obesity, insulin resistance and NAFLD(17;289)), clinically significant positive immunology titres (anti-smooth muscle antibody titre  $\geq 1:160$ (290) or anti-mitochondrial antibody titre  $\geq 1:40$ (291)) or a previous diagnosis of a persistent secondary cause for chronic liver disease. Subjects were considered to have a previous diagnosis of a secondary cause for chronic liver disease if ISD linkage revealed such a diagnosis, or if a participant report of a diagnosis was confirmed by their medical records. Subjects were excluded from calculations on the prevalence of NAFLD if data on the above measures were missing such that a secondary cause could not be excluded.

### **6.2.5 Definition of Metabolic Syndrome**

As per Adult Treatment Panel III criteria(26), subjects were considered to have the metabolic syndrome if, in addition to type 2 diabetes, they had at least two of the following: BP  $\geq$  130/85 or on antihypertensive treatment; triglycerides  $\geq$  1.7 mmol/l or taking a fibrate; HDL cholesterol  $<$  1.04 mmol/l (men) or 1.29 mmol/l (women); WC  $>$  102 cm (men) or 88 cm (women).

### **6.2.6 Statistical Analysis**

Analysis was performed using SPSS software version 14.0. Data that did not conform to the normal distribution (duration of diabetes, triglycerides and GGT) were log-transformed prior to parametric analysis. Statistical analysis included the independent t-test, chi-squared test and one-way ANOVA to compare characteristics between groups. Levels of statistical significance were reported as  $p < 0.05$  and, following Bonferroni correction,  $p < 0.003$ . Binary logistic regression analysis was used to assess the independence of variables in their association with hepatic steatosis and NAFLD. Variables included in this model were age, gender, BMI, duration of diabetes, HbA1C, systolic BP, HDL and LDL cholesterol, triglycerides and the use of metformin, sulphonylureas, thiazolidinediones, incretin mimetics and insulin. Alcohol use  $\geq$  14 units/week or previous alcohol misuse was used as a categorical variable in the model examining hepatic steatosis, but not that examining NAFLD. Results were reported as estimated odds ratios (OR) and 95% confidence intervals (CI). Sensitivity, specificity, positive and negative predictive values and ROC analysis were used to determine the utility of LFTs in the diagnosis and exclusion of hepatic steatosis.

## 6.3 Results

### 6.3.1 Subject characteristics

The characteristics of participants attending the Year 1 clinic are shown in Table 6.1. Baseline characteristics of subjects attending the Year 1 clinical examination were similar to those of the total ET2DS population suggesting that the study population attending for liver assessment remained representative of the target general population with type 2 diabetes.

Table 6.1

Characteristics of participants in the Year 1 liver clinic

Characteristic	Year 1 (n=939)
Age (years)	68.9 ± 4.2
Sex (% (n) male)	52.0 (488)
Race (% (n) Caucasian)	98.3 (923)
BMI- measured at baseline clinic (kg/m <sup>2</sup> )	31.3 ± 5.7
WC – measured at baseline clinic (cm)	106.7 ± 12.8
Duration of diabetes (years)	9.0 ± 6.4
HbA1C (%)	7.19 ± 1.06
Diet-controlled (% (n))	19.4 (182)
Oral antidiabetic agent users (% (n))	74.4 (699)
Insulin users (% (n))	15.8 (148)
Systolic blood pressure (mmHg)	138.1 ± 18.5
Diastolic blood pressure (mmHg)	74.1 ± 9.6
Total cholesterol (mmol/l)	4.15 ± 0.81
High density lipoprotein cholesterol (mmol/l)	1.23 ± 0.34
Low density lipoprotein cholesterol (mmol/l)	2.17 ± 0.68
Triglyceride (mmol/l)	1.66 ± 0.90
Statin users (% (n))	81.6 (767)
Aspirin users (% (n))	67.7 (636)
Angiotensin converting enzyme inhibitor users (% (n))	52.0 (488)
Current or ex-smokers (% (n))	60.0 (563)

Data are mean ± standard deviation or proportions, in whole cohort of participants unless stated.

### 6.3.2 Prevalence of hepatic steatosis and NAFLD

Of the 939 subjects undergoing abdominal ultrasound, data on steatosis grading was incomplete for 2 subjects and these were excluded from the analysis. Hepatic steatosis was graded as definite (Grade 3) on ultrasound examination in 56.9% (n=533) of the remaining participants. The appearance of the liver was normal (Grade 0) in 23.5% (n=220) of participants. Twenty-two subjects (2.3%) were graded as Grade 1 (in 3 of these cases it was commented that the liver was difficult to scan as it was located high up under the costal margin) and a further 16.9% (n=158) were graded as Grade 2, giving a total of 180 subjects (19.2%) who were classified as “probable normal”. In addition, 0.4% subjects (n=4) had a liver in which ultrasound identified cirrhosis – steatosis gradings in these subjects were Grade 0 in two participants, Grade 1 in one participant and Grade 2 in one participant. These findings are summarised in Figure 6.1.

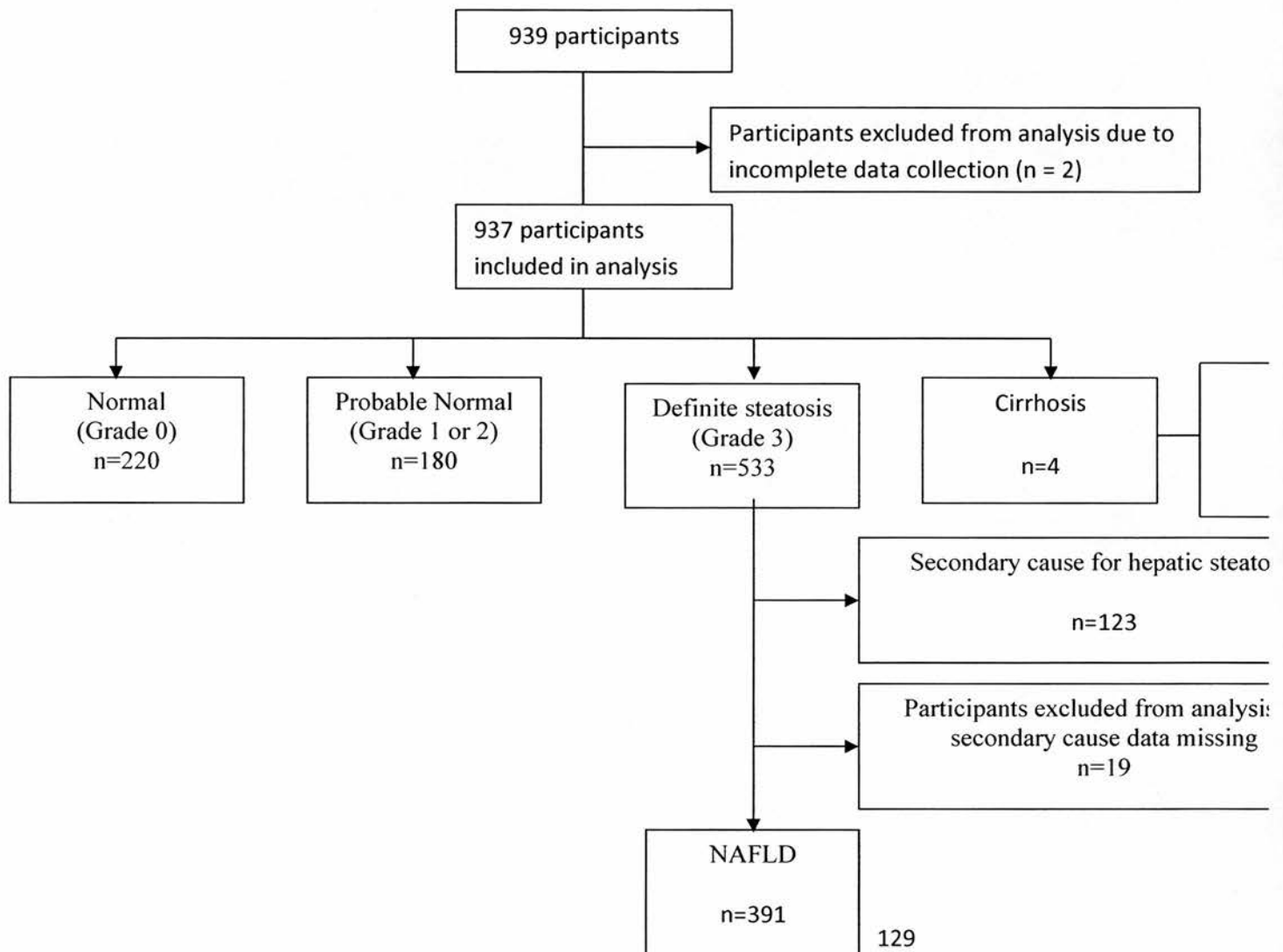
Among those with evidence of definite hepatic steatosis (Grade 3), 123 subjects had evidence of one or more secondary causes for steatosis: 78 had alcohol intake  $\geq 14$  units/week or a history of excess; 40 had used a hepatotoxic medication, as outlined above, in the previous 6 months; 1 each had serological evidence of hepatitis B and C; 3 had ferritin levels  $\geq 1000$  ug/l; 1 had anti-smooth muscle antibody titre  $\geq 1:160$ ; 1 had anti-mitochondrial antibody titre  $\geq 1:40$ ; 7 had previously-diagnosed liver disease (2 alcoholic liver disease, 1 haemachromatosis, 1 autoimmune hepatitis, 1 recurrent cholangitis, 1 biliary cirrhosis, 1 hepatic carcinoid metastases). In a further 19 subjects data were missing such that a secondary cause could not be excluded. In addition, a further 160 participants had at least one positive immunology titre  $\geq 1:40$ , which were not considered significant. Of these, most had borderline ANA or anti-smooth muscle titres ( $\leq 1:80$ ; n = 120); 40 participants had ANA titre  $\geq 1:160$ .

Using the pre-defined criteria, 391 out of 918 participants with a full dataset had definite hepatic steatosis on ultrasound grading with no secondary cause, giving a prevalence of NAFLD of 42.6% in this study population.



Figure 6.1

Flow diagram showing the gradings of hepatic steatosis and cirrhosis on ultrasound scan, and diagnosis



### **6.3.3 Clinical and biochemical associations with hepatic steatosis and NAFLD**

Physical and biochemical characteristics of study participants according to steatosis groups are shown in Table 6.2 below. On univariate analysis, participants in the group with definite steatosis (Grade 3) were significantly younger than the combined normal/probably normal groups (Grades 0, 1 and 2,  $p < 0.003$ ). BMI, WC, HbA1C, diastolic BP and triglycerides were significantly higher, and HDL cholesterol significantly lower, in the definite steatosis group. Metformin use was more common in the definite steatosis group. No significant differences in the use of other anti-diabetic agents (sulphonylureas, glitazones, incretin mimetics or insulin) were found. The higher prevalence of alcohol intake over 14 units per week in the definite steatosis group was of borderline significance ( $p = 0.003$ ).

Table 6.2

Comparison of participant characteristics across gradings of steatosis

Steatosis Grade	Grade 0	Grade 1	Grade 2	Grade 3
Characteristic	N=220	N=22	N=158	N=533
Age (years)	69.4 ± 4.2	68.5 ± 4.8	69.5 ± 4.4	68.5 ± 4.0 <sup>#</sup>
Sex (% (n) male)	59.9 (132)	40.9 (9)	49.4 (78)	50.1 (267)
BMI (baseline, kg/m <sup>2</sup> )	28.8 ± 5.18	35.1 ± 8.4	30.7 ± 4.9	32.4 ± 5.5 <sup>#</sup>
WC (cm)	102.4 ± 13.5	112.6 ±18.7	104.7 ± 11.6	108.9 ± 12.0 <sup>#</sup>
Duration diabetes (years)	9.6 ± 7.6	10.5 ± 6.0	9.8 ± 6.5	8.4 ± 5.8 <sup>*</sup>
HbA1C (%)	7.02 ± 0.97	7.15 ± 0.93	6.99 ± 0.98	7.33 ± 1.12 <sup>#</sup>
Systolic BP (mmHg)	140 ± 22	141 ± 22	136 ± 19	138 ± 17
Diastolic BP (mmHg)	73.4 ± 9.5	76.3 ± 11.2	72.1 ± 9.5	74.9 ± 9.4 <sup>#</sup>
Total cholesterol (mmol/l)	4.14 ± 0.81	4.53 ± 0.81	4.01 ± 0.75	4.17 ± 0.82
HDL cholesterol (mmol/l)	1.28 ± 0.69	1.16 ± 0.27	1.30 ± 0.35	1.19 ± 0.32 <sup>#</sup>
LDL cholesterol (mmol/l)	2.24 ± 0.70	2.57 ± 0.72	2.08 ± 0.65	2.14 ± 0.67
Triglyceride (mmol/l)	1.37 ± 0.69	1.76 ± 0.67	1.37 ± 0.63	1.86 ± 1.00 <sup>#</sup>
ALT (units/litre)	29.9 ± 10.8	26.8 ± 7.1	30.4 ± 10.0	36.4 ± 14.3 <sup>#</sup>
AST (units/litre)	28.0 ± 8.2	28.2 ± 9.7	29.9 ± 9.2	32.1 ± 11.6 <sup>#</sup>
GGT (units/litre, median (IQ range))	12 (7 – 21)	16 (9 – 34)	13 (9 – 25)	20 (12 – 34) <sup>#</sup>
Hyaluronic acid (ng/ml, median (IQ range))	45.0 (29.6 – 76.8)	53.9 (40.7 – 80.5)	46.4 (25..9 – 76.8)	45.2 (24.2 – 71.9)
Metabolic syndrome (% (n) present)	70.2 (153)	86.4 (19)	78.3 (123)	91.2 (485) <sup>#</sup>
Alcohol intake (% (n) over 14 units/week)	6.4 (14)	0.0 (0)	7.6 (12)	12.9 (69) <sup>*</sup>
Metformin use (% (n))	48.2 (106)	50.0 (11)	63.3 (100)	70.9 (378) <sup>#</sup>

\* Significant difference Grade 3 vs Grade 0/1/2 by t-test or chi-squared test, p<0.05

# Significant difference Grade 3 vs Grade 0/1/2 by t-test or chi-squared test, p<0.003

Data are mean ± standard deviation unless stated otherwise.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index BP, blood pressure; GGT, gamma-glutamyltransferase; HDL, high density lipoprotein; IQ, interquartile; LDL, low density lipoprotein; WC, waist circumference

In multivariate analysis, independent predictors of definite hepatic steatosis (compared with those classed as normal/probable normal) on ultrasound scan were BMI (OR 1.07, CI 1.04, 1.10), lesser duration of diabetes (OR for log duration diabetes 0.53, CI 0.30, 0.92), HbA1C (OR 1.35, CI 1.15, 1.59), triglycerides (OR for log triglycerides 20.76, CI 8.85, 50.85), alcohol intake  $\geq$  14 units/week (OR 3.13, CI 1.80, 5.43) and use of metformin (OR 2.19, CI 1.59, 3.00).

Similar results were obtained when those with secondary causes for liver disease were excluded from analysis. Independent predictors of NAFLD were BMI (OR 1.07, CI 1.03, 1.10), lesser duration of diabetes (OR for log duration diabetes 0.46, CI 0.25, 0.83), HbA1C (OR 1.29, CI 1.08, 1.54), triglycerides (OR for log triglycerides 17.57, CI 6.68, 46.18) and use of metformin (OR 2.25, CI 1.60, 3.17).

Of the 400 participants who had gradings of normal/probable normal liver parenchyma on ultrasound scan, 74 had at least one of the following features suggestive of possible hepatic fibrosis or cirrhosis: spleen size  $\geq$  13 cm (16 participants); HA  $>$  75 ng/ml (the upper end of normal on the laboratory reference range) in the absence of joint disease (54 participants); or platelet count  $<$   $150 \times 10^9/l$  (20 participants). If these participants were excluded from regression analysis, independent predictors of definite steatosis and NAFLD were unchanged (data not shown).

### 6.3.4 Relationship of hepatic steatosis with liver enzymes

Levels of ALT, AST and GGT were significantly higher in participants with hepatic steatosis compared with those with a normal liver on ultrasound. Mean values, however, remained within normal limits and the significant majority of subjects with hepatic steatosis had LFT values within the laboratory normal range, as is shown in Table 6.3.

Table 6.3

Comparison of LFTs between groups with hepatic steatosis and normal liver

	Normal liver (Grade 0-2) N = 400	Hepatic steatosis (Grade 3) N = 533
ALT (units/l)	29.9 ± 10.3	36.4 ± 14.3*
ALT > 50 units/l (%)	3.4	12.0*
AST (units/l)	28.7 ± 8.7	32.1 ± 11.6*
AST > 45 units/l (%)	4.4	11.1*
GGT (units/l)	13 (8 – 22)	20 (12 – 34)*
GGT > 55 units/l (%)	7.7	12.0 <sup>#</sup>

Data are mean ± standard deviation or median (interquartile range) unless otherwise stated. \*p < 0.003 for comparison between groups; <sup>#</sup>p < 0.05 for comparison between groups

Sensitivity, specificity, PPV and NPV were calculated for ALT (using both our laboratory upper limit of normal, and the previously suggested upper limits of normal of 30 units/l for men and 19 units/l for women(91)), AST and GGT. Results are shown in Table 6.4. Lowering the cut-off that defines a positive ALT result improved the sensitivity of the test, but at the expense of a significant reduction in specificity, and the NPV of a “normal” result remained suboptimal. As is demonstrated on ROC curve analysis (Figure 6.2), there is no one cut-off of ALT with high enough sensitivity and specificity to be used in clinical practice. Combining baseline and Year 1 values for ALT, AST and GGT did not improve on the performance of Year 1 values alone (data not shown).

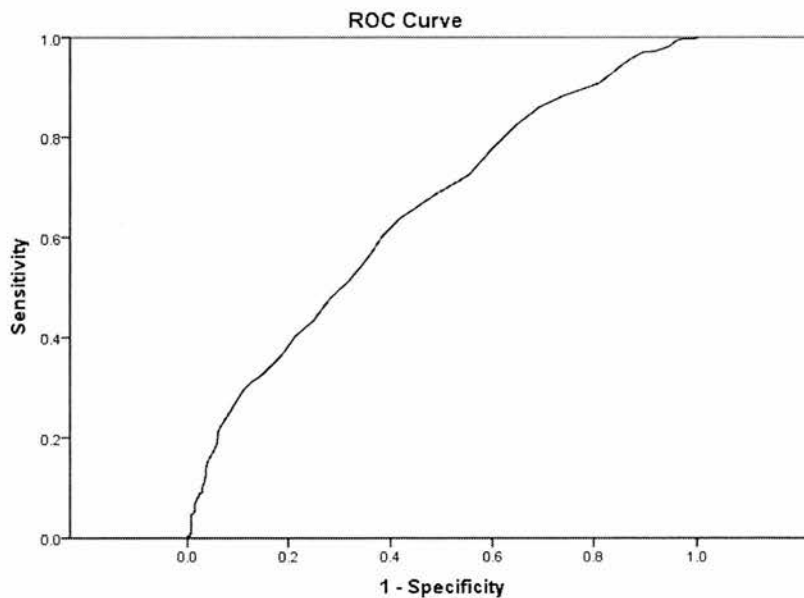
Table 6.4

Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) of LFTs in the diagnosis of hepatic steatosis. Cut-off values for a positive result are noted in brackets.

	Sensitivity	Specificity	PPV	NPV
ALT (50 units/l)	12.0 (9.4 – 15.1)	96.6 (94.1 – 98.0)	82.1 (71.4 – 89.5)	45.5 (42.2 – 48.9)
ALT (30 units/l men; 19 units/l women)	81.4 (77.8 – 84.6)	36.2 (31.6 – 41.1)	62.6 (58.9 – 66.2)	59.8 (53.3 – 65.9)
AST (45 units/l)	11.0 (8.6 – 14.1)	95.6 (93.0 – 97.3)	76.6 (65.3 – 85.2)	44.9 (41.6 – 48.3)
GGT (55 units/l)	12.0 (9.4 – 15.1)	92.3 (89.2 – 94.7)	67.4 (56.9 – 76.4)	44.4 (41.0 – 47.8)

Figure 6.2

ROC curve examining the sensitivity and specificity of ALT in the diagnosis of hepatic steatosis. AUC is 0.650.



LFTs were also compared between Groups 0, 1 and 2 using the original grading system for steatosis (Table 6.2). AST and GGT levels were statistically significantly higher in Group 2 (which contained participants who, on the original grading system, were felt to have changes suggestive of mild steatosis) than in Group 0 (the normal group), but these differences were numerically small. There was no difference in ALT or hyaluronic acid levels.

## 6.4 Discussion

This is the first study to examine the prevalence of hepatic steatosis and NAFLD in a population of people with type 2 diabetes using an ultrasound classification that had been refined by comparison with MRS and with detailed exclusion of secondary causes of steatosis. The study population included the full clinical spectrum of type 2 diabetes and included people who were being managed in the community as well as in hospital clinics.

### 6.4.1 Prevalence of hepatic steatosis and NAFLD: accuracy of diagnosis and comparison with previous studies

Previous studies have estimated that a hepatic FF on MRS of under 6.1–9% is consistent with a normal liver, whereas hepatic steatosis is associated with higher FF(63;70;73;74). Comparison of our ultrasound gradings with MRS suggested that the “definite steatosis” grading was an excellent predictor for the presence of hepatic steatosis. In contrast, considerable overlap was observed between those graded initially as having “indeterminate” or “mild” steatosis (Grade 1 or 2) and the normal group, and it was considered that these participants probably had a normal liver. Our prevalence of hepatic steatosis, at 56.9%, was therefore considerably lower than in previous studies including that of a large Italian population of patients with type 2 diabetes, in which 85.3% were considered to have hepatic steatosis(18). In view of these findings, caution should be used with regard to the prevalences reported by previous studies that have used ultrasound measurements that have been less rigorously examined for the diagnosis of hepatic steatosis.

NAFLD was the most common cause for steatosis, accounting for 76% of all cases. In comparison to previous studies, our analysis had the advantage of a more systematic identification and exclusion of secondary causes of liver disease in those with steatosis, both by the use of ISD linkage to identify previously-diagnosed chronic liver disease and by the routine measurement of autoantibody titres and ferritin in all participants with steatosis. The use of these additional measures identified a further 12 subjects with a secondary cause for hepatic steatosis, representing 2.3% of participants with steatosis and 1.3% of the study population as a whole. The systemic identification and exclusion of subjects with any possible secondary cause of steatosis from the diagnosis of NAFLD may have caused an underestimation of the prevalence of NAFLD which may in some cases have been a co-



existent pathology(22). It is recognised that the cut-offs used to define exclusions to the diagnosis of NAFLD are, to some extent, arbitrary and the prevalence will depend upon the precise definition used.

The majority of exclusions from the diagnosis of NAFLD were on the basis of excess alcohol intake. It is recognised that estimation of alcohol consumption can be unreliable, and in particular that subjects and investigators may underestimate intake. This, in turn, can lead to over-estimation of the prevalence of NAFLD. The prevalence of alcohol intake  $\geq 14$  units/week as a secondary cause for steatosis was relatively low in our study population (14.6%) when compared to a similar age band in the general population of England, in which the prevalence of alcohol intake  $\geq 21$  units/week for men and  $\geq 14$  units/week for women has been quoted as 23% and 10% respectively(292). It is possible that this reflects genuinely lower alcohol consumption in a frailer population in which use of multiple prescription medications is not uncommon. It is, however, possible that underestimation of alcohol consumption has played a role and interestingly, when participants in this study counted number of alcoholic drinks consumed over the previous week alone, a further 24 participants had intake  $\geq 14$  units.

Subjects in this study were all aged 61 to 76 years at the time of examination, and were predominantly Caucasian. Previous studies have shown that the prevalence of NAFLD increases with age (although we did not find evidence of that within our age range) and therefore it is possible that the prevalence of NAFLD in our study population was greater than that of the general diabetic population. The results may be less applicable to populations in other countries, particularly those in which the prevalence of other causes of liver disease (such as viral hepatitis) is markedly different to that in the United Kingdom.

#### **6.4.2 Clinical and biochemical correlates**

It has previously been shown that NAFLD is associated with features of the metabolic syndrome within the general population(6;19;29;150), and the same was true in this population of people with type 2 diabetes. The association of a shorter duration of diabetes with liver disease was in this study of borderline statistical significance, but has been described before (although conversely, longer duration of diabetes has also been associated

with NAFLD and more advanced liver disease(18;170)). One theory is that the greater degree of hyperinsulinaemia in early type 2 diabetes drives uptake of FFA by hepatocytes. Another possible explanation is that longer duration of diabetes is associated with hepatic fibrosis and regression of steatosis thus giving a false negative on ultrasound scanning; against this theory is the fact that duration of diabetes remained a significant inverse predictor of hepatic steatosis and NAFLD after participants with any biochemical indication of fibrosis were removed from the analysis. Similarly, the relationship of HbA1c to liver disease has varied between studies, with a positive association on univariate analysis in another large scale study in people with Type 2 diabetes but a negative result in another smaller study(171). Studies that have examined the relationship of HbA1c to progression of NAFLD have found no association(167;173). Unexpectedly, the use of metformin was associated with the presence of NAFLD, independently of BMI and glycemic control. Previous studies of the use of metformin in NAFLD have shown a positive or neutral effect in individuals(286), and it therefore seems unlikely that there is a causative link between metformin use and NAFLD here. It is possible that those participants who were on metformin had other risk factors for NAFLD that were not accounted for in the present analysis, such as inflammatory markers. Furthermore, it is possible that results were confounded by indication, i.e. participants may have been previously prescribed metformin in an attempt to treat hepatic steatosis. Finally, it is possible that the significant association between metformin and steatosis was a consequence of a type 1 statistical error.

Levels of significance were quoted for p values  $< 0.05$  and  $< 0.003$ . This second value was chosen following Bonferroni correction for the number of variables examined on univariate analysis. It is recognised that using the more stringent cut-off will reduce the chance of false positive associations, but at the expense of false negative results. This is especially true when there is a plausible biological hypothesis to link the variables under scrutiny.

#### **6.4.3 Utility of LFTs in the diagnosis of hepatic steatosis**

Although ALT, AST and GGT levels were significantly higher in the group with definite steatosis compared with the normal/probable normal group, mean values remained within the normal range. This is in keeping with previous studies carried out within the general population that have found the diagnostic accuracy of liver enzymes in predicting hepatic steatosis to be low(93). A study performed since the above work was carried out, in a

Brazilian population of patients within a tertiary referral centre for diabetes, produced very similar results for a cut-off of ALT of 36 units/l (sensitivity 52%, specificity 76%, PPV 81% and NPV 41%). In the present study, even when the upper limit of normal of ALT was reduced to lower previously-suggested levels the NPV remained low. There were no cut-off values that discriminated between normal and steatotic livers and, importantly, low levels of hepatic enzymes did not preclude overt hepatic abnormality demonstrated by ultrasonography. To take account of intra-individual variation in LFT values, NPVs of consistently normal LFTs were examined (i.e. in individuals who had “normal” values of ALT, AST or GGT at both the baseline and Year 1 clinics); disappointingly this did not improve on the ability of normal LFTs to predict a normal liver on ultrasound.

At present, liver screening in Type 2 diabetes is commonly carried out using conventional LFTs and it is of concern that normal levels of liver transaminases and GGT are found in almost 90% of those with hepatic steatosis, suggesting that most cases will not be identified by current methods.

The results were mixed when levels of liver enzymes were compared between the “normal” and “probable normal (originally “mild steatosis”)” groups, with statistically significantly higher levels of AST and GGT, but not ALT or hyaluronic acid, in the “probable normal” group. Consistent evidence that the “probable normal” group is a subgroup with liver disease other than steatosis which is picked up on ultrasound (such as inflammation or fibrosis) is therefore lacking.

#### **6.4.4 Methodological limitations**

One limitation of this study is that subjects did not have a liver biopsy and histological examination, the gold standard technique for identifying steatosis; performance of this invasive procedure would neither have been feasible nor ethical in a population study of this magnitude. It was recognised that some participants with a normal USS could have undiagnosed hepatic fibrosis and thus be at the severe end of the spectrum of NAFLD. Of note, any misclassified cases would tend to underestimate the prevalence of NAFLD and reduce rather than magnify any differences in clinical associations between groups. Furthermore, re-analysis excluding those participants with a normal liver USS but other

evidence of possible fibrosis revealed similar results to the main analysis. A further limitation is that only approximately one fifth of patients invited to attend the baseline clinic from the Lothian Diabetes Register did so. Significantly, however, analysis revealed that this population was representative of that invited in terms of duration of diabetes, HbA1C and treatment with insulin.

It is acknowledged that while data on most variables were gathered concomitantly with hepatic ultrasound examination, BMI was calculated one year previously during the baseline examination. It is therefore possible that the results of the regression analysis are less robust than they would have been with fully contemporaneous data collection. It seems likely, however, that changes in BMI over one year would in most cases be small and would be unlikely to significantly affect the results of analysis.

#### **6.4.5 Conclusions**

In conclusion, in assessing the prevalence of NAFLD in people with type 2 diabetes, this study has advantages of robust ultrasound gradings, systematic exclusion of other causes for liver disease, and a relatively large, unselected population. In the future it is possible that the association of liver disease with other features of the metabolic syndrome could be used to target screening for NAFLD. Current screening methods that rely on liver enzyme measurement alone are almost certainly inadequate. Further research is required to ascertain whether the risk of progression of NAFLD to fibrosis and cirrhosis in patients with type 2 diabetes in the clinical setting is as high as that predicted – if so, this would provide further impetus towards improved screening to aid early diagnosis so that people might be eligible for entry into clinical trials, screening for complications and ultimately new therapies.

### **Analysis 3: Prevalence and markers of advanced liver disease**

#### **7.1 Introduction**

##### **7.1.1 Prevalence of advanced NAFLD: relationship to Type 2 diabetes**

NAFLD encompasses a spectrum of liver disease from simple steatosis to steatohepatitis, fibrosis and, ultimately, cirrhosis. Cross-sectional data suggest that in populations of patients with NAFLD the prevalence of advanced fibrosis (bridging fibrosis or cirrhosis) may be of the order of 9 – 14%, with a higher proportion of the population having lower-grade fibrosis(96;174;175). In longitudinal studies, it has been estimated that 8% of patients with simple steatosis progress to hepatic fibrosis, with a higher proportion progressing when steatohepatitis is evident at presentation(99;181;293). These figures are, however, based on biopsy results from highly-selected patient populations and there is little consensus on how they might relate to patients in whom biopsy is not clinically justified. Although type 2 diabetes is an established risk factor for the progression of NAFLD(64;209), few studies have examined the prevalence of advanced liver disease in this population. Existing evidence is often indirect or has originated from small studies or those undertaken in highly selected populations. In one population-based study, the risk of death from liver cirrhosis was 2.5 times higher in people with type 2 diabetes compared with the general population(211). In another study of 132 patients with NAFLD (44 of whom had diabetes) who had undergone liver biopsy in a tertiary referral centre, 25% of those with diabetes had cirrhosis, compared to 10% of the non-diabetic cohort(67).

##### **7.1.2 Non-invasive markers of advanced liver disease**

Screening for liver disease in Type 2 diabetes generally involves the measurement of standard LFTs alone. Not only are these tests insensitive markers of hepatic steatosis, as has been described in detail in Chapters 1 and 6(63;96), but it has also been previously demonstrated that the progression of NAFLD to fibrosis can occur without significant

perturbation of liver enzymes(64;96). It has been suggested that lowering the upper limit of normal of ALT to 30 units/l in men and 19 units/l in women may improve sensitivity of this measure in detecting NAFLD(91), but this has not been widely adopted. It is therefore likely that many cases of NAFLD in the people with diabetes are not identified and progressive liver disease is often missed. However, few data are available on the applicability of LFTs to diagnose hepatic fibrosis in people with diabetes.

Other approaches have been taken in the pursuit of a reliable non-invasive method of distinguishing those patients who have significant fibrosis. HA is a component of the extracellular matrix, whose production is increased, and degradation by the liver decreased, in hepatic fibrosis. Several small studies have suggested that cut-off values of 42-50 ng/ml give optimal sensitivity and specificity in determining the presence of significant fibrosis(102-105). Higher levels may be associated with increasing severity of liver disease and increased specificity in diagnosing fibrosis and cirrhosis. One study found both sensitivity and specificity in detecting hepatic fibrosis to be in excess of 90% using a HA cut-off value of 149 ng/ml(106). A further study suggested that a level > 100 ng/ml had a 78% specificity and 83% sensitivity for predicting cirrhosis, with specificity being raised to 96% if a cut-off of 300 ng/ml was used(107). One potential confounder is that levels of HA can also be raised in patients with active joint disease(294;295).

Routinely-available markers that are predictive of fibrosis have been combined into scoring systems. The BAAT score assigned one point for each of increased age, BMI, ALT and triglycerides, and while a score of 2 gave 71% sensitivity and 80% specificity in detecting septal fibrosis, a score of 3 gave 14% sensitivity but 100% specificity(177). The BARD score allocated one point for each of increased BMI and the presence of diabetes, and two points for AST:ALT ratio greater than 0.8 – with a cut-off of 2 points, the PPV and NPV of determining severe fibrosis were 43% and 96% respectively(196). The NAFLD fibrosis score, using a formula combining age, BMI, the presence of diabetes, AST:ALT ratio, albumin and platelet levels, classified 75% of participants low risk or high risk groups for severe fibrosis, with a low cut-off (below -1.455) giving a NPV of 93% and a high cut-off (above 0.676) a PPV of 90%(198).

Ultrasound scanning has diagnostic accuracy of 80-86% in the diagnosis of cirrhosis or extensive fibrosis in chronic liver disease(119) and can detect consequences of portal hypertension such as increased spleen size. A low platelet count is a well established marker of splenomegaly and the PSR has been shown to be a reliable predictor of oesophageal varices in people with cirrhosis(123), and can therefore be used as a surrogate marker of portal hypertension.

HCC is a well-recognised complication of hepatic cirrhosis. Diabetes is an established risk factor for HCC and in one population-based study, diabetes was associated with around a 2.5-fold increase in incidence of HCC(209), independent of alcohol intake and viral hepatitis. The prevalence of HCC in the general population of people with Type 2 diabetes, however, remains unclear.

### **7.1.3 Study aim**

The present study aimed to estimate the prevalence of fibrosis, cirrhosis and HCC, using a range of different markers and scoring systems, in this randomly-selected population of people with type 2 diabetes (the Edinburgh Type 2 Diabetes Study (ET2DS)), and to analyse the effectiveness of LFTs in screening for liver disease in this population.

## 7.2 Methods

### 7.2.1 Participants

The study population for this analysis comprised the 939 participants of the Year 1 liver study of the ET2DS, as described in Chapters 4 and 6. To summarise, subjects recorded as having type 2 diabetes mellitus, aged 60 – 74 years, were selected at random into sex and 5-year age bands from the Lothian Diabetes Register, a computerised database which contains details of over 20,000 patients with type 2 diabetes living in Lothian, Scotland and includes both patients attending hospital and those seen only in primary care. Study participants have been shown previously to be representative of all those randomly selected to participate and therefore of the target population of older men and women with type 2 diabetes living in the general population(282).

### 7.2.2 Procedures

Subjects attended a specially established research clinic, based in the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh, at baseline and after one year (Year 1). At Year 1, participants attended, after a 4-hour fast, for an ultrasound examination of abdomen and venous blood sampling.

All ultrasound examinations were performed by a single ultrasonographer as previously described(281;296). Evidence of cirrhosis and hepatic masses were sought systemically and spleen length was measured. Participants were also given an overall liver grading based on a subjective measurement of the severity of steatosis, and validation of the gradings for hepatic steatosis with <sup>1</sup>H MRS has previously been described in detail in Chapter 5.

Alcohol intake, use of hepatotoxic medications (amiodarone, isoniazid, methotrexate, tamoxifen and glucocorticoids) within the previous six months and history of joint disease were determined by questionnaire. Those participants with evidence of hepatic steatosis or abnormal blood tests of liver function had further tests performed for liver disease performed including serology for Hepatitis B and C, ANA, anti-smooth muscle antibody, anti-mitochondrial antibody, ferritin and alpha fetoprotein (AFP). In addition, all participants



(including those with normal liver and LFTs) seen after a certain date had these same screening blood tests performed (n=644). Triglycerides and HDL cholesterol were measured at Year 1. BMI, platelets and HA had been previously measured at the baseline clinic.

Participants who had significant abnormalities on hepatic USS or blood tests were referred for standard follow-up within the National Health Service. Those with focal lesions on USS had these reviewed by a consultant radiologist and received further imaging as necessary, and those with raised AFP > 20 kU/l were referred for review in a liver clinic.

Data on previous diagnoses of chronic liver disease were collected from participants by questionnaire at Year 1. In addition, data on liver diagnoses were obtained at baseline from discharge summaries via record linkage at the ISD of NHS Scotland.

Upper limits of normal on the laboratory reference range of bilirubin (Bi), ALT, AST and GGT were 18  $\mu\text{mol/l}$ , 50 units/l, 45 units/l and 55 units/l respectively. Analysis was also carried out using lower upper limits of normal for ALT – 30 units/l for men and 19/units/l for women(91).

### **7.2.3 Definition of NAFLD, hepatic fibrosis, HCC and PSR**

NAFLD was defined as the presence of definite (Grade 3) hepatic steatosis on ultrasound scan in the absence of a secondary cause for hepatic steatosis. In addition, for this study, the definition was widened to include people with markers of fibrosis but no evidence of hepatic steatosis on ultrasound, in acknowledgement of the fact that steatosis can regress in patients with significant fibrosis. Secondary causes were defined as alcohol consumption  $\geq 14$  units/week or participant report of a current or previous problem with alcohol excess, use of hepatotoxic medication (glucocorticoids, isoniazid, methotrexate, amiodarone and tamoxifen) within the six months prior to the Year 1 clinic, positive hepatitis B or C serology, ferritin > 1000  $\mu\text{g/l}$ , clinically significant positive immunology titres (anti-smooth muscle antibody titre  $\geq 1:160$  or anti-mitochondrial antibody titre  $\geq 1:40$ ) or a previous diagnosis of a secondary cause for chronic liver disease (alcoholic liver disease, autoimmune

hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis or liver metastases)(296). Subjects were considered to have a previous diagnosis of a secondary cause for chronic liver disease if ISD linkage revealed such a diagnosis, or if a participant report of a diagnosis was confirmed by their medical records.

Hepatic fibrosis was defined on the basis of HA levels – values of over 100 ng/ml, in the absence of arthritis, were taken as evidence of definite fibrosis and possible cirrhosis(107). A cut-off of 50 ng/ml was taken to indicate possible fibrosis(102-105); values over 75 ng/ml are also reported as this is the upper limit of normal on our laboratory reference range.

HCC was defined, in accordance with accepted guidelines(297), by one of the following: two imaging techniques showing a focal lesion over 2cm in diameter with arterial hypervascularisation; one imaging technique showing a focal lesion over 2cm in diameter in association with AFP levels >400 ng/ml (484 kU/l); suggestive histology following biopsy of a lesion. PSR was defined as the ratio of platelet count (/mm<sup>3</sup>) to spleen diameter (mm)(123).

#### **7.2.4 Fibrosis clinical scoring systems**

Participant scores on established scoring systems (BAAT, BARD and NAFLD Fibrosis scores) were calculated. The BAAT(177) and BARD(196) scoring systems are outlined above. NAFLD Fibrosis Score was calculated as previously published(198). Scores of 2-4 on both the BAAT and BARD scores were considered to be predictive of hepatic fibrosis. On the NAFLD Fibrosis Score, a score of >0.676 was considered to be predictive of fibrosis, and a score of -1.455–0.676 was indeterminate for fibrosis. In view of the fact that these systems have been developed to assess the severity of NAFLD alone, they were used only in those participants in whom another secondary cause for liver disease had been excluded.

#### **7.2.5 Statistical analyses**

Statistical analysis was performed using SPSS software version 14.0 (SPSS Inc., Illinois, USA). Data not conforming to a normal distribution (in this study GGT measurements) were log-transformed prior to parametric analysis. Statistical analysis included the one-way

analysis of variance and chi-squared test to compare biochemical characteristics between groups. PPV and NPV were used to analyse the utility of LFTs in predicting hepatic fibrosis.

## 7.3 Results

### 7.3.1 Prevalence of markers of fibrosis, cirrhosis and HCC

Baseline characteristics of the 939 participants, aged 61 – 75 years, who attended the Year 1 clinic are described in Chapter 6. Hepatic cirrhosis on ultrasound scan was found in four members of the study population, a prevalence of 0.4%. Portal hypertension, defined as a PSR <909, was present in 10 participants (1.1%), including one participant who had cirrhosis on ultrasound scan. A total of 876 participants were fully assessed for secondary causes of liver disease. Of these, 663 participants had no secondary cause identified, and in this subgroup findings suggestive of fibrosis or cirrhosis would be presumed secondary to NAFLD. Hepatic cirrhosis on ultrasound scan was found in one person (0.2%) in this subgroup and PSR < 909 in four other people (0.6%).

The prevalence of definite hepatic fibrosis, defined as HA level > 100 ng/ml in the absence of joint disease, was 5.7% in the entire study population of 939 participants. The corresponding figures for HA level > 50 ng/ml and > 75 ng/ml were 23.6% and 12.2% respectively. 0.5% of the population had HA level > 300 ng/ml. An additional 20.6% of participants had HA levels > 50 ng/ml but also had evidence of arthritis. Of the 13 participants with evidence of cirrhosis on ultrasound scan or a low PSR, 4 (30.8%) had HA levels 50-100 ng/ml and a further 6 (46.2%) had HA levels > 100 ng/ml.

In the subgroup of participants with no secondary cause for liver disease, 40 participants (6.1%) had HA > 100 ng/ml in the absence of joint disease, 166 participants (25.2%) had HA > 50 ng/ml, 86 participants (13.0%) had HA > 75 ng/ml and 4 participants (0.6%) had HA > 300 ng/ml.

The prevalence of hepatic fibrosis was similar among participants who had evidence of hepatic steatosis on ultrasound and among those without steatosis. In the group with hepatic steatosis, the prevalence of hepatic fibrosis (HA > 100 ng/ml in the absence of joint disease) was 5.6%; in the group without steatosis this figure was 5.7% .

Two participants had definite HCC, a prevalence in the study population of 0.2%. In one case this was confirmed on histology following biopsy of a 3.7cm liver mass on a background of cirrhosis seen on USS, in association with raised AFP levels of 40 kU/l. A second participant had a very high AFP (2712 kU/l); no mass was seen on USS, but appearances on magnetic resonance imaging were supportive of a diagnosis of HCC. Neither participant had a secondary cause for liver disease, giving a prevalence of 0.3% in the group with possible NAFLD.

### **7.3.2 Comparison of clinical fibrosis scoring systems with radiological and biochemical markers**

The three clinical scoring systems for prediction of fibrosis secondary to NAFLD were used as alternative means of calculating prevalence of fibrosis in the subgroup with no secondary cause for liver disease. The BARD score was positive in 92.6% compared to 79.3% for the BAAT score, while 16.4% of the subgroup had a positive NAFLD Fibrosis Score, and in a further 66.8% this score was indeterminate.

The only participant in this subgroup to have cirrhosis on ultrasound scan had positive BARD, BAAT and NAFLD Fibrosis scores. When the prevalence of positive fibrosis scores were compared across three groups based on HA measurements (HA < 50 ng/ml (or arthritis present); HA 50 – 100 ng/ml in the absence of arthritis; and HA > 100 ng/ml in the absence of arthritis) the only positive correlation was with the NAFLD Fibrosis Score with the prevalence of a positive score rising from 13.7% to 21.3% to 42.5% in the three HA groups respectively ( $p < 0.001$ ). Conversely, 72.5% of those with a positive NAFLD Fibrosis Score had a HA measurement greater than 50 ng/ml.

### **7.3.3 Comparison of tests of liver function with markers of fibrosis**

Standard tests of liver function were compared between three groups according to HA level in participants with no secondary cause for liver disease (Table 7.1). There was a significant rise in mean AST, but not mean ALT, through the groups, but mean levels of ALT, AST and GGT remained within the laboratory normal reference range in all three groups. Similarly, mean levels of ALT, AST and GGT remained within the normal range in all three groups

when participants were divided according to NAFLD Fibrosis Score (< -1.455; -1.455–0.676; and >0.676: data not shown). The PPV and NPV of ALT and GGT in predicting fibrosis as diagnosed using HA levels and the NAFLD Fibrosis Score are presented in Table 7.2. The PPV and NPV of proposed new upper limits of normal of ALT (>30 units/l for men, >19 units/l for women) are also shown in Table 7.2.

Table 7.1

Comparison of ALT, AST and GGT between participants grouped according to HA level.

	HA < 50 ng/ml (n = 494)	HA 50-100 ng/ml (n = 126)	HA >100 ng/ml (n = 40)	Cirrhosis on USS (n = 1)
<b>ALT (units/l)</b>	33.3 ± 12.5	34.0 ± 13.5	34.9 ± 14.5	38.0
<b>ALT &gt; 50 units/l (No. (%))</b>	34 (6.9)	9 (7.1)	5 (12.5)	0 (0)
<b>AST (units/l)</b>	30.1 ± 9.9	31.5 ± 9.4	35.3 ± 12.6*	62.0
<b>AST &gt; 45 units/l (No. (%))</b>	29 (5.9)	8 (6.3)	4 (10.0)	1 (100)
<b>GGT (units/l)</b>	16.0 (10.0 – 27.0)	14.0 (18.8 – 22.0)	18.5 (11.5 – 35.0)	43
<b>GGT &gt; 55 units/l (No. (%))</b>	36 (7.3)	7 (5.6)	7 (17.5)*	0 (0)

ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma glutamyltransferase, HA: hyaluronic acid.

Data are mean ± standard deviation or median (interquartile range) unless stated otherwise.

\*Significant differences between the groups stratified according to HA level by one-way ANOVA, Kruskal-Wallis or chi-squared tests ( $p < 0.05$ )

Table 7.2

Positive and negative predictive values of standard LFTs (ALT (using standard and alternative upper limits of normal) and GGT) in the diagnosis of fibrosis secondary to NAFLD, as defined using HA levels or the NAFLD Fibrosis Score.

	ALT (cut-off 50 units/l)		ALT (alternative cut-offs <sup>†</sup> )		GGT (cut-off 55 units/l)	
	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
<b>HA*</b> (cut-off 50 ng/ml)	29.2	75.1	25.1	74.8	28.0	75.1
<b>HA*</b> (cut-off 100 ng/ml)	10.4	94.3	6.0	93.7	14.0	94.6
<b>NAFLD Fibrosis Score#</b>	15.9	13.6	20.5	10.3	22.9	14.6

\*Participants with arthritis were grouped with those with a HA level below the cut-off

#PPVs are predictive of a NAFLD Fibrosis Score > 0.676; NPVs are predictive of a NAFLD Fibrosis Score < -1.455.

<sup>†</sup>Upper limit of normal taken as 30 units/l for men and 19 units/l for women

## 7.4 Discussion

Detection of advanced liver disease using non-invasive methods is challenging. This study examines the prevalence of markers of hepatic fibrosis and cirrhosis, and their relationship to standard tests of liver function, in a large randomly-selected cohort of older people with Type 2 diabetes. The prevalence of hepatic fibrosis using HA was 5.7% and that of ultrasound-diagnosed cirrhosis under 1%. The use of LFTs as a screening tool missed the majority of cases of fibrosis predicted by raised HA levels.

### 7.4.1 Prevalence of advanced liver disease in type 2 diabetes: comparison with previous studies

Previous reports have estimated and predicted the prevalence of hepatic fibrosis, cirrhosis and HCC secondary to NAFLD in people with Type 2 diabetes to be higher than in the non-diabetic population – but results have often either been extrapolated from other data or based on results from relatively highly-selected patient populations. In comparison, the present study has the advantage that participants were selected at random from a register of all people in the South-East of Scotland who had Type 2 diabetes, and therefore represented the entire spectrum of this condition. It is the first study to include people with diabetes who are receiving their routine review in the community as well as those in the secondary care setting. These factors are likely to have contributed to the lower rates of severe fibrosis and cirrhosis seen here, relative to previous studies of patients with clinical indications for liver biopsy seen within tertiary referral centres(96;174;175). It is also possible that the high rate of use of anti-diabetic agents in the population, particularly metformin, may have had a disease modifying effect on NAFLD and contributed to the lower than expected rates of fibrosis and cirrhosis.

The prevalence of HCC in our cohort was 0.2%, a figure which is comparable to that previously found on screening both a general population in Italy (prevalence of 0.7%)(298). It is also in keeping with the only previous large-scale study, carried out in Taiwan(299), that has looked at prevalence of HCC in a subgroup of people with type 2 diabetes, in which the prevalence was 0.1%. This population, unlike ours, had a high prevalence of viral hepatitis. In contrast, prevalence of HCC is higher in populations with known cirrhosis, and in one small-scale study the prevalence in an obese group with cryptogenic cirrhosis was 30%(300).



Longitudinal studies in people with NAFLD (diagnosed on biopsy) have found a variable incidence of HCC, ranging from no cases during a follow-up time of 11 years in a population with simple steatosis alone, to 2.4% of the research population in a study of NASH over a maximum follow-up period of 21 years(178;181).

#### **7.4.2 Use of non-invasive markers of fibrosis and portal hypertension**

HA was the main marker of fibrosis used in this study, with cut-offs for fibrosis and cirrhosis defined as in previous reports(102-105;107). Liver biopsy, the gold standard, would have been neither feasible nor ethical, and by contrast HA measurement is relatively non-invasive. Transient elastography was not available to us, and is not without its drawbacks in a largely overweight patient population. We attempted to improve specificity of HA by excluding participants with arthritis (another cause of raised HA) from the fibrosis categories, and also by using a high cut-off (100 ng/ml) for our definition of definite evidence of fibrosis. While we can be reasonably confident that the proportion of the study population with fibrosis is over 5.7% (those with HA levels > 100 ng/ml, without joint disease), it is possible that the prevalence of fibrosis was lower than the 26% predicted by HA levels > 50 ng/ml. It is of interest that, contemporaneously with the submission of our data for publication, a study examining prevalence of advanced liver disease in a population of Brazilian Type 2 diabetic patients, based within a hospital clinic, was published(173). In this study, participants (who had already been screened for secondary causes of liver disease) with hepatic steatosis were automatically referred for the gold standard liver biopsy: between 6.5 and 10% of the entire study population had advanced fibrosis; and 2.2% had cirrhosis. Results were therefore broadly in keeping with the findings of the present study.

The only clinical scoring system to positively correlate with HA measurements was the NAFLD Fibrosis Score, which predicted definite fibrosis in 16.4% of participants with no secondary cause for liver disease. A further 66.8% of subjects had an indeterminate score. This is a much higher proportion than in the original paper, which suggested that such patients should be considered for liver biopsy(198). The BARD and BAAT scores both estimated that a very high percentage of participants in this study had fibrosis but in view of the emphasis on age, BMI and diabetes in these scoring systems, this could have been anticipated. Interestingly, when the BARD score was validated in a subgroup with type 2 diabetes in the initial study, the AUC was lower than in the group as a whole (0.53 vs 0.80).

This supports the concept that these more simple scoring systems, while useful in the general population of patients with NAFLD, may be less applicable to populations of people with type 2 diabetes. The risk of their indiscriminate use is that they may identify large numbers of patients for biopsy without any definitive evidence of a high prevalence of advanced liver disease.

A low platelet count is a well-established consequence of hypersplenism, and the PSR has previously been validated as a marker of portal hypertension (123). This validation was, however, carried out in patients with known cirrhosis and it is possible that its use is less applicable in our study population. In particular it is possible an alternative diagnosis may have underpinned the finding of hypersplenism in some of our participants and this may have contributed to the lack of overlap seen in our measures of cirrhosis and PSR.

#### **7.4.3 Analysis of data in the absence of the accepted gold standard**

As discussed above, the validity of the results in this chapter has been weakened by the fact that the use of the accepted gold standard measure for advanced liver disease (biopsy) was not possible. This is a problem that is by no means unique to the current work, and it is not uncommon to encounter situations where a gold standard cannot be used in all participants, where the gold standard is imperfect or where there is no gold standard(301;302).

Several mechanisms have been used to overcome these drawbacks. One group of solutions that is particularly relevant to the current scenario is the use of multiple test results to construct a reference standard outcome(301;303). There are three recognised means of combining the results of tests in this context. The first (the “composite reference standard”) is to use a predefined rule to classify patients, a procedure that is transparent and easy to use, but is perhaps simplistic and may result in a relatively high proportion of misclassified patients. The second (“panel diagnosis”) is to utilise a panel of experts to combine the data and reach a consensus on individual participants, which may produce a more flexible approach that more closely reflects personal concepts of the target condition. The third (“latent class analysis”) is to use statistical modelling based on actual data, which has the disadvantage that the condition is not defined in a clinical fashion and so it may not be clear

how the results relate to clinical practice. A secure evidence base for each approach is currently lacking.

The use of any one of the above methods can be envisaged as having a contribution to make to the understanding of the data presented. The use of a pre-defined rule to classify patients, such as by a certain combination of HA results and ultrasound findings, would be relatively straightforward, whereas the panel approach, while potentially providing a more accurate representation of individuals within the population, might be prohibitively time-consuming in a study of this size. Statistical modelling of data has previously been shown to be rewarding in this setting, such as in the NAFLD Fibrosis Score. Future work might focus on validating these approaches in the current setting, perhaps by using future clinical outcome as the validation measure.

#### **7.4.4 Comparison of LFTs with biochemical markers of hepatic fibrosis**

Although significant increases were noted in the values of AST through the groups with increasing evidence of liver damage, mean values of all three LFTs examined remained within normal limits. This is consistent with previous studies which have shown that progression to cirrhosis can occur without major perturbation in the LFT values(64). Although the NPVs for ALT and GGT were high in predicting lower HA levels, these values are affected by the very low population prevalence of HA-diagnosed fibrosis. The use of lower values for the upper limit of normal of ALT did not materially change the results. Currently, liver screening in Type 2 diabetes is carried out using conventional LFTs alone, and our data suggest that such an approach misses the majority of cases of hepatic fibrosis.

#### **7.4.5 Limitations of this study**

The main limitation of this study (as detailed above) was the use of surrogate markers of advanced liver disease rather than histological examination, the acknowledged gold standard investigation. However, to perform liver biopsy routinely in a study population of essentially healthy volunteers would have been unethical due to its invasive nature. Subjects in this study were all aged 61-76 years at the time of examination, and were predominantly Caucasian. Previous studies have shown that the prevalence of NAFLD and its progression

to fibrosis and cirrhosis increases with age. It is therefore possible that the results from our study population may not be representative of those from other age ranges. Furthermore, at baseline, only approximately one fifth of people invited to attend from the Lothian Diabetes Register did so – but analysis has suggested that this population was representative of that invited, most importantly in terms of duration of diabetes, HbA1c and treatment with insulin.

#### **7.4.6 Conclusion**

In conclusion, this study provides an estimate of prevalence of hepatic fibrosis in a large population of older people with Type 2 diabetes. There are challenges in detecting advancing liver disease, and current screening methods using conventional LFTs are almost certainly inadequate. Further work is required to refine identification of these patients, particularly in those in whom liver biopsy would be difficult to justify. This may require a panel of biochemical markers, for example the ELF panel or cytokeratin-18, or a radiological method such as transient elastography. Future work might also focus on how best to combine the multiple data points available, in order to produce a more meaningful categorisation of participants into those with and those without more advanced liver disease, by utilising mechanisms such as panel diagnosis.

### **Analysis 4: Association between hepatic steatosis/NAFLD and clot lysis dynamics in Type 2 diabetes**

#### **8.1 Introduction**

NAFLD encompasses a spectrum of disease from simple steatosis to NASH, fibrosis and cirrhosis. It has been shown to be a risk factor for cardiovascular disease both in the general population(205;233) and in patients with type 2 diabetes(18;222). Emerging risk factors for cardiovascular disease (CVD) in this context include a systemic pro-thrombotic tendency and subclinical inflammation.

##### **8.1.1 Clot formation and lysis dynamics: relationship to pro-thrombotic mediators, diabetes and NAFLD**

One area of interest is inter-individual differences in clot structure and lysis. Previous studies have shown that individuals at high cardiovascular risk have higher clot density, altered clot morphology and longer clot lysis times compared with controls(239-242). Similar alterations in clot structure and lysis times have also been seen in individuals with type 1 and type 2 diabetes, and have been correlated with glycaemic control(243;244). Study of clot dynamics in patients with NAFLD has been limited. One small study compared clotting kinetics, measured using thomboelastography of whole blood samples, in 28 patients with NAFLD (either diagnosed on ultrasound or on biopsy) and 22 control subjects, and demonstrated higher clot strength and reduced clot lysis in the group with NAFLD, independent of the presence of diabetes or features of the metabolic syndrome(275).

The formation and stability of the platelet-rich clot depends on the establishment of a fibrin mesh, which in turn is dependent on a complex interaction of components of the coagulation

cascade and other plasma proteins. Fibrinogen(239;240;247), PAI-1(239;241) and complement C3(243;250), a central component of the innate immune system, concentrations have been shown to contribute to variation in clot morphology and clot lysis in non-diabetic and diabetic populations. They have also been individually associated with an increased risk of cardiovascular disease(252;266;276;304;305). There have been conflicting results when associations with liver disease have been examined(274;277;279;280). The relationship of these pro-thrombotic mediators to clot kinetics has not been studied in relation to NAFLD.

Hepatic triglyceride accumulation and subsequent hepatocyte damage has been shown to be associated with increased cytokine release. TNF $\alpha$  and IL-6 production from nearby visceral fat further perpetuates this vicious cycle(271;272). Studies in patients with NAFLD have revealed higher serum concentrations of inflammatory markers, including TNF $\alpha$ , IL-6 and CRP, compared to controls(270;273;274). There are several mechanisms by which the increase in cytokines associated with NAFLD may affect the risk of cardiovascular disease(236); of interest here is the possible contribution to increased hepatic production of pro-coagulant factors such as fibrinogen and the antifibrinolytic protein PAI-1.

### **8.1.2 Study aim**

The primary aim of the current study was to examine the association of hepatic steatosis and NAFLD with clot formation and lysis dynamics and inflammatory mediators in a large, well-defined population of people with Type 2 diabetes. We hypothesised that participants with hepatic steatosis and NAFLD would have a greater prothrombotic tendency (higher clot density and longer clot lysis time) and higher levels of inflammatory mediators.

## 8.2 Methods

### 8.2.1 Participants

The 939 participants in the Year 1 liver clinic, as described in Chapters 4 and 6, are the subject of this further investigation.

### 8.2.2 Liver ultrasound examination and clinical examination

Subjects attended the year 1 research clinic after a 4-hour fast for an ultrasound examination of abdomen as described in previous chapters. The liver was graded for markers of hepatic steatosis using established criteria(306-308) including a bright hepatic echo pattern (compared with the echo response of the right kidney), increased attenuation of the echo beam and the presence of focal fatty sparing. Following validation of ultrasound gradings with <sup>1</sup>H MRS, the gold-standard non-invasive method of quantifying hepatic fat content, subjects were graded as hepatic steatosis present or absent(296;309). Further investigations were carried out to detect secondary causes for liver disease as outlined in Chapter 6.

At the time of recruitment into the study, participants attended a baseline examination which included measurement of demographic and anthropometric variables (age, gender, BMI), fibrinogen, platelets and HA. At year 1 further measurements were performed including BP, LFTs (bilirubin, ALT, AlkP and GGT), HbA1c, triglycerides, high-density cholesterol (HDL) and low-density cholesterol (LDL).

### 8.2.3 Definitions of liver disease

Three groups were defined: the *steatosis group* had definite hepatic steatosis of any cause on ultrasound; the *NAFLD group* was a subgroup of the steatosis group with no secondary cause for liver disease; the *normal group* had no evidence of hepatic steatosis or cirrhosis on ultrasound scanning. Secondary causes for steatosis were alcohol consumption  $\geq 14$  units/week(10) or participant report of a current or previous problem with alcohol excess, use of hepatotoxic medication(17) (glucocorticoids, isoniazid, methotrexate, amiodarone and tamoxifen) within the six months prior to the Year 1 clinic, positive hepatitis B or C serology, ferritin concentration  $\geq 1000$  ug/l (milder hyperferritinemia is known to be

associated with obesity, insulin resistance and NAFLD(17;289)), clinically significant positive immunology titres (anti-smooth muscle antibody titre  $\geq$  1:160(290) or anti-mitochondrial antibody titre  $\geq$  1:40(291)) or a previous diagnosis of a persistent secondary cause for chronic liver disease. The secondary exclusions from NAFLD are strict and it is possible that some participants who have NAFLD as a primary or coexistent diagnosis may be excluded from this definition (for example those with relatively modestly elevated alcohol intake below that traditionally felt to cause alcoholic liver disease). Therefore analysis has been performed both with and without exclusions from the steatosis group.

#### **8.2.4 Analysis of clot formation and lysis**

Blood samples were taken without a tourniquet after a 4h fast. The first 5 mls were used for clinical tests and subsequent 9 mls of whole blood was added to 1 ml of 0.9% citrate and samples were spun down within 2 hours of collection with plasma stored at  $-40^{\circ}\text{C}$  until analysis. Clot turbidity and lysis measurements were conducted as previously described (310). Briefly, 25  $\mu\text{l}$  of plasma samples was added to 75  $\mu\text{L}$  lysis mix composed of assay buffer (50  $\text{mmol}^{-1}$  Tris, 100  $\text{mmol}^{-1}$  NaCl, pH 7.4) and 83 ng/ml tPA (Technoclone, Vienna, Austria), followed by the addition of 50  $\mu\text{L}$  of activation mix containing assay buffer, 0.03 U/mL thrombin (Calbiochem) and 7.5 mmol/L  $\text{CaCl}_2$ . Plates were shaken and read at 340 nm every 12 sec for 60 min then every 2 minutes for 9 hours in an EL<sub>x</sub>-808 IU ultramicroplate reader (BIO-TEK Instruments INC, USA). A software application was used to analyse the data and variables were recorded as follows:

*Lag time* is the time until sufficient protofibrils have formed to enable lateral aggregation, effectively the time taken until the beginning of the exponential rise in maximum absorbance.

*Clot formation time* is the time from start of lateral aggregation to full clot formation. Both clot density and effectiveness of fibrinolysis contribute to this measurement.

*Maximum absorbance* is a measure of fibrin network density and fibre thickness, clot turbidity being directly proportional to the cross-sectional area of fibrin fibres

*Clot lysis time* represents time from full clot formation to 50% lysis.



### **8.2.5 Measurement of prothrombotic and inflammatory mediators**

Fibrinogen levels were assessed on plasma samples using the automated Clauss assay (MDA-180 coagulometer, Organon Teknika). PAI-1 and C3 levels were determined using ELISA kits (Invitrogen, Paisley, UK, and GenWay Biotech, San Diego, USA respectively). CRP was assayed using a high-sensitivity immunonephelometric assay. TNF $\alpha$  and IL-6 levels were determined using high-sensitivity ELISA kits (R&D Systems, Oxon, UK).

### **8.2.6 Statistical analysis**

Statistical analysis was performed using SPSS software version 14.0. The student t-test, Mann-Whitney and chi-squared tests were used to compare variables between groups. Spearman's correlation coefficient was used to determine correlations between variables, many of which were not normally distributed. Data that did not conform to the normal distribution were log-transformed prior to parametric analysis. As multiple univariate comparisons were carried out a Bonferroni calculation was made for each analysis, and significance at  $p < 0.003$  levels (in addition to  $p < 0.05$ ) was quoted where possible. For Spearman correlations, computer programming constraints meant that significance at  $p < 0.01$  was the lowest possible. Linear regression analysis was used to examine the independence of variables in their association with clot lysis. Independent contributors were presented as standardised correlation coefficient ( $\beta$ ). Tolerance measures did not suggest significant multicollinearity. Variables included in the model were age, gender, presence of NAFLD/steatosis (vs. no steatosis), BMI, triglycerides, duration of diabetes, HbA1c, metformin use, LDL, current smoker at baseline, fibrinogen, PAI-1, platelets, C3 and CRP.

## 8.3 Results

### 8.3.1 Participant characteristics

Participant characteristics, grouped according to the presence of hepatic steatosis or the subgroup with NAFLD, are presented in Table 8.1.

Table 8.1

Characteristics of participants.

	Normal liver (n=406)	Hepatic steatosis (n=533)	NAFLD (n=391)
Age (years)	69.4 ± 4.3	68.5 ± 4.0 <sup>*</sup>	68.5 ± 4.0 <sup>*</sup>
Gender (% (n) male)	54.4 (221)	50.1 (267)	46.8 (183) <sup>*</sup>
BMI (kg/m <sup>2</sup> )	29.9 ± 5.5	32.4 ± 5.5 <sup>*</sup>	32.6 ± 5.7 <sup>*</sup>
Duration diabetes (yrs)	8.0 (4.3 – 13.0)	7.0 (4.0 – 11.0) <sup>*</sup>	7.0 (4.0 – 11.0) <sup>*</sup>
HbA1c (%)	7.0 ± 0.1	7.3 ± 1.1 <sup>*</sup>	7.3 ± 1.1 <sup>*</sup>
Diet-controlled (% (n))	22.4 (91)	17.1 (91) <sup>*</sup>	15.9 (62) <sup>*</sup>
Systolic BP (mm Hg)	138.4 ± 20.6	137.9 ± 16.7	137.1 ± 15.8
Diastolic BP (mm Hg)	73.0 ± 9.6	74.9 ± 9.4 <sup>*</sup>	74.6 ± 9.3 <sup>*</sup>
Total cholesterol (mmol/l)	4.1 ± 0.8	4.2 ± 0.8	4.1 ± 0.8
HDL cholesterol (mmol/l)	1.3 ± 0.4	1.2 ± 0.3 <sup>*</sup>	1.2 ± 0.3 <sup>*</sup>
LDL cholesterol (mmol/l)	2.2 ± 0.7	2.1 ± 0.7	2.1 ± 0.7
Triglyceride (mmol/l)	1.2 (0.9 – 1.7)	1.6 (1.3 – 2.2) <sup>*</sup>	1.6 (1.3 – 2.2) <sup>*</sup>
Metformin users (% (n))	54.2 (220)	70.9 (378)	72.4 (283) <sup>*</sup>
Sulphonylurea users (% (n))	30.5 (124)	31.5 (168)	30.7 (120)
Thiazolidinedione users (% (n))	17.0 (69)	17.8 (95)	19.9 (78)
Insulin users (% (n))	17.0 (69)	14.8 (79)	15.3 (60)
Aspirin users (% (n))	67.2 (273)	68.1 (363)	70.3 (275)
ACE inhibitor users (% (n))	52.5 (213)	51.6 (275)	50.4 (197)
Current smokers (% (n))	14.0 (57)	11.3 (60)	10.2 (40)

Data are expressed as mean ± standard deviation or median (interquartile range) unless otherwise stated. <sup>\*</sup>p < 0.05 compared with Normal group

### 8.3.2 Relationship of hepatic steatosis and NAFLD with clot formation and lysis dynamics

Clot lysis time was significantly higher in participants with hepatic steatosis compared to the normal group (657 (498 - 850) vs 597 (468 - 774) seconds,  $p = 0.002$ ). A similar result was obtained when the NAFLD sub-group was compared to the normal group. There were no significant differences between the steatosis/NAFLD and normal groups in lag time, time to clot formation or maximum absorbance. These results are detailed in Table 8.2.

Table 8.2

The association of hepatic steatosis and NAFLD with clot formation and lysis dynamics, inflammatory markers and components of the coagulation cascade

	Normal liver (n = 406)	Steatosis (n = 533)	NAFLD sub-group (n = 389)
LT (seconds)	522 (451 – 679)	538 (464 – 680)	539 (468 – 692)
CFT (seconds)	538 (462 – 624)	528 (464 – 624)	528 (468 – 624)
MA (au)	0.33 ± 0.13	0.32 ± 0.11	0.32 ± 0.11
CLT (seconds)	597 (468 – 774)	657 (498 – 850)**	672 (492 – 888)**
PAI-1 (pg/ml)	439.9 (203.9 – 868.5)	796.1 (379.0 – 1531.4)**	755.1 (367.4 – 1446.0)**
Fibrinogen (mg/ml)	3.70 ± 0.77	3.56 ± 0.70*	3.58 ± 0.69*
C3 (g/l)	1.29 ± 0.48	1.38 ± 0.49*	1.36 ± 0.51*
CRP (mg/l)	1.44 (0.66 – 3.35)	2.14 (0.99 – 4.58)**	1.86 (0.89 – 4.27)*
TNFα (pg/ml)	1.06 (0.68 – 1.63)	1.09 (0.71 – 1.63)	1.07 (0.68 – 1.62)
IL-6 (pg/ml)	2.64 (1.67 – 4.26)	2.90 (2.06 – 4.40)*	2.78 (2.02 – 4.19)
Platelets (x10 <sup>9</sup> /l)	254 ± 70	260 ± 68	263 ± 66

Data are expressed as mean ± standard deviation or median (interquartile range) unless otherwise stated. LT, lag time; CFT, clot formation time; MA maximum absorbance; CLT, clot lysis time. \* $p < 0.05$  when compared with the normal group; \*\* $p < 0.003$  when compared with the normal group.

### **8.3.3 Relationship of hepatic steatosis and NAFLD with pro-thrombotic and inflammatory mediators**

PAI-1 and C3 levels were significantly higher, and fibrinogen levels significantly lower, in the group with steatosis and the sub-group with NAFLD when compared to the normal group (Table 8.2 above). CRP was also significantly higher in the steatosis and NAFLD groups compared with the normal group, but there was no significant difference in TNF $\alpha$  between the groups. IL-6 was significantly higher in the steatosis group compared with the normal group, but the result did not reach statistical significance when the NAFLD group was compared with the normal group. Platelet number was not significantly different between the groups.

### **8.3.4 Determinants of clot formation and lysis dynamics**

Univariate analysis to analyse the associations of clot formation and lysis dynamics (clot formation time, maximum absorbance and clot lysis time) was carried out separately on people with a normal liver on ultrasound and on those with NAFLD. The results are presented in Table 8.3. Fibrinogen and CRP levels were consistently correlated with all three clot dynamic measures. C3 was correlated with clot lysis time in both groups, but with maximum absorbance in the normal group only. PAI-1 was associated with clot lysis time in the NAFLD group but not in the normal group. Lipids (HDL and LDL cholesterol, and triglycerides) were significantly associated with clot lysis time in the normal group (inverse association of HDL cholesterol) but not in the NAFLD group.

In a separate analysis, BMI was significantly correlated with PAI-1, fibrinogen, C3, CRP and IL-6 in the normal group; in the NAFLD group the correlations with PAI-1, fibrinogen, CRP and IL-6 remained but here BMI was *not* associated with C3 (data not shown). HbA1c was significantly associated with C3, but not the other mediators, in both groups.

Table 8.3

Association of clinical characteristics, prothrombotic and inflammatory mediators with clot formation and lysis dynamics in the study population. Data are Spearman rho values from bivariate correlations.

	Clot formation	Maximum absorbance	Clot lysis
<b>Normal liver</b>			
Age (years)	0.00	0.07	0.09
Duration diabetes (years)	<b>0.10</b>	<b>0.10</b>	0.00
HbA1c (%)	0.00	0.09	<b>0.13</b> <sup>**</sup>
BMI (kg/m <sup>2</sup> )	0.07	<b>0.11</b>	<b>0.10</b>
Triglycerides (mmol/l)	0.04	-0.04	<b>0.10</b>
HDL cholesterol (mmol/l)	-0.07	-0.07	<b>-0.12</b>
LDL cholesterol (mmol/l)	-0.10	0.00	<b>0.16</b> <sup>**</sup>
PAI-1 (pg/ml)	<b>0.18</b> <sup>**</sup>	0.10	0.08
Fibrinogen (mg/ml)	<b>0.32</b> <sup>**</sup>	<b>0.45</b> <sup>**</sup>	<b>0.15</b> <sup>**</sup>
C3 (g/l)	0.049	<b>0.14</b>	<b>0.28</b> <sup>**</sup>
CRP (mg/l)	<b>0.16</b> <sup>**</sup>	<b>0.31</b> <sup>**</sup>	<b>0.20</b> <sup>**</sup>
TNF $\alpha$ (pg/ml)	<b>0.13</b> <sup>**</sup>	0.00	0.05
IL-6 (pg/ml)	<b>0.13</b>	<b>0.16</b> <sup>**</sup>	0.07
<b>NAFLD</b>			
Age (years)	-0.06	-0.04	-0.07
Duration diabetes (years)	0.03	0.04	-0.03
HbA1c (%)	0.02	<b>0.11</b>	<b>0.19</b> <sup>**</sup>
BMI (kg/m <sup>2</sup> )	<b>0.16</b> <sup>**</sup>	<b>0.18</b> <sup>**</sup>	<b>0.15</b> <sup>**</sup>
Triglycerides (mmol/l)	0.08	-0.08	0.05
HDL cholesterol (mmol/l)	0.09	<b>0.14</b> <sup>**</sup>	0.02
LDL cholesterol (mmol/l)	-0.02	0.07	0.08
PAI-1 (pg/ml)	<b>0.17</b> <sup>**</sup>	<b>0.14</b> <sup>**</sup>	<b>0.14</b> <sup>**</sup>
Fibrinogen (mg/ml)	<b>0.34</b> <sup>**</sup>	<b>0.38</b> <sup>**</sup>	<b>0.14</b> <sup>**</sup>
C3 (mg/l)	-0.02	0.02	<b>0.26</b> <sup>**</sup>
CRP (mg/l)	<b>0.16</b> <sup>**</sup>	<b>0.17</b> <sup>**</sup>	<b>0.14</b> <sup>**</sup>
TNF $\alpha$ (pg/ml)	<b>0.13</b> <sup>**</sup>	0.03	0.05
IL-6 (pg/ml)	0.07	<b>0.22</b> <sup>**</sup>	0.07

Significant factors in bold. \*p < 0.05, \*\*p < 0.01

The association of fibrinogen with use of medication was investigated, in a post-hoc analysis. Participants on metformin had significantly lower levels of fibrinogen than participants who did not take this drug ( $3.59 \pm 0.70$  vs  $3.72 \pm 0.77$  mg/ml,  $p < 0.05$ ). However the lower levels of fibrinogen persisted (statistically significantly in the steatosis vs normal analysis, and as a trend in the NAFLD vs normal analysis) in the steatosis/NAFLD group even after participants on metformin were excluded from analysis. There were no significant differences in fibrinogen levels between users and non-users of thiazolidinediones, aspirin, statins or angiotensin converting enzyme inhibitors. The lack of association between hepatic steatosis/NAFLD and clot formation or maximum absorbance remained after adjustment for fibrinogen levels and metformin use.

As hepatic steatosis and NAFLD were significantly associated with clot lysis time, this relationship was examined in more detail. On multivariate analysis, the association between NAFLD and clot lysis time was attenuated by the separate addition of each of ambient glucose, BMI, WC, PAI-1, C3, fibrinogen and inflammatory markers (CRP, TNF $\alpha$  and IL-6), but a statistically significant association remained. However, the association was lost when full adjustment was made for clinical and biochemical variables (Table 8.4). Independent predictors of clot lysis time were C3, HbA1c and LDL cholesterol. NAFLD was not an independent predictor of clot lysis time in this model. This model accounted for 11.3% of the variance in clot lysis time (adjusted  $R^2 = 0.113$ ).

Table 8.4

Multivariate analysis of the association between clot lysis, NAFLD and clinical and biochemical characteristics.

	Standardised $\beta$ coefficients			
	Model 1	Model 2	Model 3	Model 4
NAFLD	<b>0.105*</b>	0.072	0.056	0.059
Age	-0.054	-0.046	-0.021	-0.018
Sex	<b>0.143*</b>	<b>0.100*</b>	<b>0.093*</b>	0.078
ln(PAI-1)		0.068	0.047	0.056
C3		<b>0.224*</b>	<b>0.216*</b>	<b>0.219*</b>
Fibrinogen		0.016	0.011	0.022
ln(CRP)		0.055	0.028	0.015
Platelets		0.076	<b>0.080*</b>	0.072
ln(duration diabetes)			<b>-0.083*</b>	-0.074
Ambient glucose			0.022	0.020
HbA1c			<b>0.117*</b>	<b>0.108*</b>
Metformin use			-0.034	-0.018
BMI			0.069	0.075
ln(triglycerides)			-0.027	-0.032
Current smoker				-0.005
LDL cholesterol				<b>0.094*</b>

Model 1: NAFLD + age/sex

Model 2: NAFLD + age/sex + prothrombotic and inflammatory mediators

Model 3: NAFLD + age/sex + prothrombotic and inflammatory mediators + diabetes/metabolic syndrome variables

Model 4: NAFLD + age/sex + prothrombotic and inflammatory mediators + diabetes/metabolic syndrome variables + traditional cardiovascular risk factors

Significant values in bold. \*p < 0.05

## 8.4 Discussion

In this large, randomly-selected, population of older people with type 2 diabetes, the presence of hepatic steatosis or NAFLD was associated with a longer *ex vivo* clot lysis time and higher levels of PAI-1 and C3. Following multivariate adjustment for these, and other clinical characteristics, the independent association of NAFLD and clot lysis time was lost. We therefore hypothesise that NAFLD affects clot lysis dynamics via increased hepatic synthesis of pro-coagulant factors, thus contributing to a systemic pro-thrombotic tendency. Another possible explanation is that NAFLD is a marker of visceral adiposity and increased adipocyte production of hypofibrinolytic mediators.

### 8.4.1 Relationship of NAFLD with clot dynamics: role of pro-thrombotic mediators

We have shown higher C3 levels in participants with NAFLD, and C3 is itself independently associated with clot lysis time, both in the present study and previous reports(243;251). We therefore hypothesise that the production of C3 by the liver is increased in NAFLD and this is one mechanism by which NAFLD prolongs clot lysis time and predisposes to cardiovascular disease. It is of interest that in the NAFLD group (unlike in the normal group) C3 was not significantly correlated with BMI; this adds weight to a possible direct role of hepatocytes, rather than an indirect association via visceral adiposity. A previous study, comparing children with type 1 diabetes with controls, showed higher C3 concentration and incorporation into clots in the diabetic subjects(243). This suggested a role of C3 that is intrinsic to the coagulation process rather than an indirect effect via its inflammatory properties. It is important to note, however, that in the present study C3 was positively correlated with maximum absorbance in the normal group but not in the NAFLD group, which might argue against a significant change in clot structure via C3 in this group.

One further mechanism is via an increase in PAI-1 production from the liver, with higher levels of PAI-1 seen in the NAFLD group in the present study, and the inclusion of PAI-1 attenuating the relationship between NAFLD and clot lysis time on regression analysis. In addition, PAI-1 was significantly correlated with clot lysis time in the NAFLD group but not in the normal group. Animal studies have suggested that after fat accumulation in the liver, hepatocytes become a more important site of PAI-1 production(311); in humans, a positive



correlation between the presence of hepatic steatosis and liver PAI-1 expression has previously been demonstrated(278). In the present study, PAI-1 was significantly positively correlated with BMI in both the NAFLD and normal groups, and so the possibility that steatosis and NAFLD are markers of increased adipocyte production of this protein cannot be excluded.

Contrary to expectation, NAFLD was associated with lower fibrinogen levels than were seen in participants with a normal liver on ultrasound. One possible explanation was the differing use of metformin in the two groups. We have previously documented a positive association between NAFLD and metformin use(296), most likely due to confounding by indication, and in the present cohort participants taking metformin had statistically significantly lower levels of fibrinogen. It might therefore have been possible that the lower levels of fibrinogen in the NAFLD group are due to the higher rate of metformin use in this group(312). Against this theory is the fact that the association between lower levels of fibrinogen and steatosis/NAFLD persisted after participants on metformin were removed from the analysis. Another possibility is that the time lag between measurement of fibrinogen and assessment of liver for NAFLD may have played a role. Against this hypothesis, fibrinogen showed the expected positive correlations with clot formation markers, which were measured simultaneously with liver assessment. The negative correlation of fibrinogen and NAFLD therefore remains unexplained but may itself be responsible for the fact that NAFLD was not associated with longer clot formation time and higher clot density. Despite the higher levels of fibrinogen in the normal group, clot lysis time was significantly shorter suggesting that this protein plays a marginal role in the susceptibility of the clot to lysis.

Although it is possible that increased hepatic production of prothrombotic factors is responsible for the results documented, an alternative hypothesis is that hepatic steatosis is merely a marker for abdominal obesity, insulin resistance, and associated higher production of acute-phase proteins from tissues other than the liver such as adipocytes and mononuclear cells. The fact that BMI (and WC) attenuated rather than abolished a significant association between the presence of NAFLD and clot lysis time would go against this. Although we did not perform analysis of production of C3 and PAI-1 at a tissue level, our conclusions that an increase in hepatic production is at least partly responsible for the higher plasma levels is supported by previous studies as outlined above.

The pathogenesis of NAFLD is both affected by and contributes to a pro-inflammatory milieu. In our analysis, CRP was significantly associated with clot lysis time and with higher levels of prothrombotic mediators. The relative influence of inflammation at the level of the liver, as opposed to inflammatory activity elsewhere remains unclear, and it cannot be excluded that NAFLD is a marker of (rather than a significant contributor to) a systemic inflammatory and prothrombotic state.

#### **8.4.2 Association of glycaemic control with clot lysis time**

Higher HbA1c was an independent predictor of clot lysis time, whereas ambient glucose level at the time of venepuncture was not. Our data therefore suggests that longterm glycaemic control is more important than short term perturbations in glucose. The observation that higher HbA1c predicts longer clot lysis time is in keeping with a previous study that has shown higher clot lysis time in patients with, versus those without, diabetes(243;313). An independent association between higher HbA1c and the presence of NAFLD in this cohort has been demonstrated in Chapter 5 and this is likely due to the increase in insulin resistance associated with hepatic steatosis. One hypothesis is that poorer glycaemic control promotes formation of clots that have a denser, less porous structure and are more resistant to fibrinolysis(244), and that this effect is partially mediated by glycation of the fibrinogen molecule. Alternatively, it is possible that glucotoxicity associated with poorer glycaemic control indirectly affects clot composition via an increase in C3 as has been previously postulated.

#### **8.4.3 Strengths and limitations of the current study**

The present study has a number of strengths. In particular, our cohort of participants is large, representative and well-characterised. There are few previous studies that have examined the association of NAFLD with clot dynamic and inflammatory mediators and those that exist are small and often in selected populations. Furthermore, we have used robust methodology in identifying participants with hepatic steatosis and NAFLD including validation of our ultrasound measure with MR spectroscopy and stringent exclusion of participants with any possible secondary cause for liver disease.

Limitations of this study include its cross-sectional design, which restricts conclusions that can be drawn regarding causality. Participants were diagnosed with NAFLD on the basis of ultrasound scanning rather than on biopsy and histological examination – biopsy would have been neither feasible nor ethical in a study population of this size. The effect of misclassification would be to weaken the conclusions drawn. Our population was aged between 61 and 76 years at the time of the study and is therefore representative of many patients seen within diabetic clinics - it is however possible, although unlikely, that our conclusions cannot be generalised to a younger population. It was not possible to quantify insulin resistance formally – clamp studies would not have been feasible in a population of this size and models such as the HOMA-IR model are not accurate in patients with Type 2 diabetes who are on antihyperglycaemic medication. We have not examined genetic polymorphisms underlying structural and functional differences in the components of the coagulation cascade, or the expression of genes at a cellular or tissue level, and it likely that such analysis would add further depth to the observations made on the basis of plasma concentration alone.

#### **8.4.4 Conclusions**

In conclusion, we have demonstrated an association between NAFLD and impaired clot lysis time in this population of people with type 2 diabetes, and postulate that this is one mediator of the increased cardiovascular risk previously observed in such patients. Further research into the association of NAFLD and clot lysis dynamics with longitudinal cardiovascular and mortality outcomes will help to further define these relationships.

### Discussion and Conclusions

#### 9.1 Discussion and Conclusions

##### 9.1.1 Use of ultrasound to diagnose hepatic steatosis (Chapter 5)

The main finding in this study was that an initial ultrasound grading suggesting an indeterminate (Grade 1) or mildly steatotic (Grade 2) liver was, after comparison with the non-invasive gold standard MRS, more in keeping with normality than steatosis. Initial “normal” or “severe steatosis” gradings were found to be consistent with normality and steatosis respectively. Intra-observer variation in ultrasound gradings was minimal for the sonographer who performed gradings for the study as a whole, but higher and statistically significant for a registrar trainee in radiology. Inter-observer variability was higher than intra-observer variability, but non-significant when gradings of the sonographer were compared with those of a consultant radiologist.

The implications for the current study were that participants with an “indeterminate” or “mild steatosis” grading were essentially grouped with those with a “normal” grading and were excluded from a diagnosis of hepatic steatosis and NAFLD. A valid inference is that results from previous studies that have used an unvalidated ultrasound measure should be questioned, as quoted prevalence rates may represent an over-estimate of the true prevalence. Future research that utilises an ultrasound measure should strongly consider validating gradings.

It is accepted that the gold standard used in the current study was MRS rather than the “true” gold standard of biopsy. Biopsy would not have been considered ethical in an essentially healthy population. In addition, MRS may have advantages over biopsy in terms of proportion of liver sampled. In a large meta-analysis, sensitivity and specificity of MRS versus biopsy were 88.5% and 92% respectively, and it is difficult to know whether the

relatively minimal deficit was due to limitations of MRS (such as sampling variability) or to sampling variability and inter-observer variability in histological assessment(78).

Intra- and inter-observer variation in ultrasound gradings is an area of concern both clinically and in large-scale research studies. There is an element of subjectivity in gradings that appears to preclude exact concordance with MRS. Further study in this area might focus on development of a quantitative ultrasound measure, using computer software to calculate differences in echogenicity between the liver and right kidney for example. Another approach would be to move to using MRS as the standard method for ascertaining hepatic steatosis in population studies, and this scanning modality has already been used in a large-scale study in the general population in the USA(63). Currently cost (and indeed the inability to scan the most obese members of the population) is a significant barrier to routine adoption of MRS in such studies, but this may change in the future.

### **9.1.2 Prevalence and clinical correlates of hepatic steatosis and NAFLD in Type 2 diabetes (Chapter 6)**

The primary aim of this phase of the ET2DS was to accurately define prevalence of hepatic steatosis and NAFLD in a large population of people with Type 2 diabetes. The respective prevalences of 56.9% and 42.6% are considerably lower than those quoted in previous studies, and it is likely that this was due to validation of the ultrasound measure used and rigorous exclusion of those with a secondary cause for steatosis from the diagnosis of NAFLD(18). A second finding was that BMI, HbA1c, triglycerides and lesser duration of diabetes were independently associated with the presence of hepatic steatosis and NAFLD. Thirdly, the diagnostic accuracy of liver enzymes in this context was relatively poor.

The study population of the ET2DS was designed to be representative of older people with Type 2 diabetes within the Lothian region of Scotland. Representativeness data suggest that this was achieved, but it is possible that such analysis does not test subtle differences between people who chose to take part in an epidemiological study compared to those who were not willing or able. It is possible that results cannot be generalised to younger people with diabetes, but it should be remembered that the 60 – 75 age group makes up a significant proportion of the Type 2 diabetic population as a whole and results are therefore of interest.

The most accurate measure of population prevalence would come from studying an entire population, potentially via opportunistic screening (with informed consent) at the time of routine diabetes care, and one previous study captured data on the significant majority of patients with Type 2 diabetes attending a hospital clinic(18). This, however, would be difficult when the population in question is very large, and particularly when care is undertaken at a number of sites including hospital outpatient departments and general practitioner practices.

The criteria that defined secondary causes of hepatic steatosis (and thus exclusion from the diagnosis of NAFLD) are worthy of further discussion. The approach in this study was to define exclusions strictly, with the intention of characterising a population that we could feel confident had NAFLD. In particular, it is a strength that the rarer causes of liver disease were systematically sought by biochemical testing and use of record linkage. The limitations of this approach are that it is likely that a proportion of those labelled as having a secondary cause for steatosis (e.g. those with a modest increase in alcohol intake outwith the range usually felt to be consistent with alcoholic liver disease) actually had NAFLD. Furthermore, it is appreciated that NAFLD can co-exist with other liver diseases and this would not have been picked up using the present methodology. There is no one accepted definition of the secondary causes of steatosis and this has led to heterogeneity between previous studies. These limitations underline the shortcomings of the current means of defining NAFLD, which is currently principally a diagnosis of exclusion. Future work might focus on building a definition of NAFLD that is based on positive features such as those identified on multivariate analysis in the current study population – obesity, Type 2 diabetes/glycaemic control and hypertriglyceridaemia – in addition to clear exclusions such as significant alcohol excess.

The observation that traditional LFTs are poorly sensitive in the diagnosis of hepatic steatosis and NAFLD is in keeping with previous studies carried out in the non-diabetic population. Use of previously-suggested lower cut-offs for upper limit of normal of ALT resulted in improved sensitivity but at the expense of specificity(91). There are three conclusions that can be drawn from this. Firstly, in the context of Type 2 diabetes, any ALT, AST or GGT level above the laboratory reference range is highly indicative of liver pathology and should be followed up with further investigation. Secondly, LFTs in the

middle and upper parts of the normal range do not preclude overt abnormality on ultrasound and should therefore not be viewed as screening tools for hepatic steatosis and NAFLD. Thirdly, ALT within the normal range can be viewed as a continuum marker of risk, and it is therefore conceivable that it could be used in combination with other clinical markers, perhaps as part of a scoring system, to define “at risk” persons who might then go on to have confirmatory tests. Future research might examine this further.

In conclusion, this study has confirmed the importance of accurate diagnosis of hepatic steatosis and NAFLD in population studies of prevalence. Further studies examining prevalence of steatosis and NAFLD in a group with Type 2 diabetes unselected as to age would be valuable. Moreover, the use of routinely-available clinical parameters to target people for ultrasound screening should be investigated further. At present a single biochemical parameter that identifies those with steatosis remains elusive.

### **9.1.3 Prevalence and markers of advanced liver disease (Chapter 7)**

The use of HA as a surrogate marker identified a prevalence of hepatic fibrosis of 5.7% in this entire population of older people with Type 2 diabetes. Corresponding figures for prevalence of ultrasound-diagnosed cirrhosis and HCC were 0.4% and 0.2%. When participants with a secondary cause for liver disease, in whom fibrosis could be assumed to be secondary to NAFLD, comparable prevalences of fibrosis, cirrhosis and HCC were 6.1%, 0.2% and 0.3% respectively. Once again routine LFTs performed poorly in the detection of people with markers of advanced liver disease.

This study is one of the first to attempt to investigate the prevalence of advanced liver disease in a population of people in whom liver biopsy would not be routinely justified. It might have been anticipated that the prevalence of advanced liver disease would be higher in this high risk population. However this supposition is based on data from selected patient populations in which biopsy has been clinically indicated. It is perhaps surprising that the prevalence of fibrosis appears to be less than that found in biopsy studies in obese individuals undergoing bariatric surgery (in whom the prevalence was of the order of 10%), but these patients were morbidly obese often with Type 2 diabetes and this combination is likely to account for the difference(97;176). It is of interest that results are comparable to

those from another study in people with Type 2 diabetes that is contemporaneous to the present analysis(173).

The significant limitation of this study was the use of surrogate markers to define advanced liver disease. The gold standard, histological examination of liver tissue, was not considered to be feasible or ethical in this study population. The use of HA has been justified by previous reports that examined its diagnostic accuracy in comparison with biopsy; it is, however, recognised that the PPV of this test will be affected by the prevalence of the condition in the population under investigation. We attempted to improve specificity of the test by using a relatively high cut-off to define fibrosis and by excluding people with arthritis from the diagnosis. Nonetheless, results may be viewed as interesting baseline work to be confirmed by subsequent investigation. Future studies might utilise a combination of markers of fibrosis such as the ELF panel (biochemical markers of matrix turnover, including HA, TIMP 1 and P3NP), along with imaging assessment of hepatic stiffness using transient elastography or magnetic resonance elastography. In addition, markers of NASH (as opposed to fibrosis specifically), such as cytokeratin-18 may be examined.

The proportion of people identified as requiring further investigation for advanced liver disease (ostensibly biopsy) by available clinical risk scores was found to be much higher than in the original studies performed in the non-diabetic population. Interestingly, the most complex of the three scoring systems used in the current study (the NAFLD Fibrosis Score) designated the majority of participants as being at indeterminate rather than high risk; this was the only model to correlate with HA levels here. Currently there is not enough information available to formally assess the diagnostic accuracy of the clinical risk scores in the present Type 2 diabetic population. It would, however, seem reasonable to presume that the PPV of all three scores will be less in the general Type 2 diabetic population than in a population of patients that have had clinical features of liver disease significant enough to justify biopsy, and indeed a previous study has suggested that the utility of the BARD score is less in a population with Type 2 diabetes than in the general population. In the absence of a routinely-available biochemical marker of high diagnostic accuracy, a reliable clinical risk scoring system would be valuable in selecting patients for biopsy, but it is clear that in order to be clinically useful such a score would have to be developed within the population at which it is targeted, in this case in a general population of people with Type 2 diabetes. An



inherent difficulty with future research into this issue is the lack of a concrete gold standard, assuming that biopsy is ethically unjustifiable. As discussed in Chapter 7, one approach to this difficulty would be to validate a construct reference standard against clinical outcomes (including liver failure, complications of cirrhosis and death); the ET2DS provides a potential forum for this as 5-year (and beyond) data will become available in the near future.

In conclusion, the use of surrogate markers of hepatic fibrosis is justifiable in the current study population and has resulted in a prevalence of hepatic fibrosis that is in keeping with the only other known estimate. Future work should focus on refining this estimate with use of a battery of non-invasive markers, and more generally on validating such markers along with clinical scoring systems in the Type 2 diabetic population. Longitudinal studies will be valuable in defining the rate of and risk factors for progression in this population, Clarity on this point may aid a decision on the usefulness of screening for different stages of NAFLD.

#### **9.1.4 Relationship of hepatic steatosis and NAFLD with clot formation and lysis dynamics, and pro-thrombotic mediators (Chapter 8)**

The main finding of this analysis was that the presence of hepatic steatosis and NAFLD was associated with a longer clot lysis time and higher levels of the pro-thrombotic mediators PAI-1 and C3. Following multivariate adjustment for these mediators and clinical characteristics, the independent relationship of NAFLD and clot lysis time was lost.

The principal hypothesis is that hepatic steatosis and NAFLD contribute to a prothrombotic and inflammatory milieu that in turn contributes to variation in clot lysis time. It is not possible to say with any certainty whether the steatotic liver is directly responsible for the increased production of biochemical mediators (i.e. synthesis and release from hepatocytes), or whether it is a marker of systemic inflammation and visceral obesity, with adipocytes and mononuclear cells playing a significant role in production. Further investigation of mRNA and protein synthesis at a tissue level would clarify this.

An unexpected finding was the positive association between hepatic steatosis/NAFLD and lower fibrinogen levels. This has not been fully explained and further work to determine if this is a reproducible finding and factors behind it would be valuable.

An area of particular interest is whether the increased clot lysis time might moderate the previously-observed relationship between NAFLD and cardiovascular disease. The present analysis has not explored this and a further study might focus on elucidating these associations, perhaps by analysing the effect of NAFLD and clot lysis dynamics at baseline on longitudinal outcomes of cardiovascular disease and mortality. As clot formation and lysis are likely to have most effect on acute events such as myocardial infarction, it would be constructive to analyse these events separately from the effects of long-term atherosclerosis such as angina.

#### **9.1.5 Conclusion**

The current work adds to our understanding of the prevalence and risk factors for NAFLD within the Type 2 diabetic population, but several interlinking questions of clinical importance remain incompletely answered. In particular, it remains debated whether we should screen for NAFLD, which patients should be routinely screened and how this should be carried out.

The evidence, both here and in the work of other authors, is persuasive that a significant percentage of people with NAFLD in the context of Type 2 diabetes and obesity develop hepatic fibrosis. Despite this, the proportion that go on to display associated morbidity and mortality in the form of clinically apparent liver disease may be smaller than previously postulated. Longitudinal follow-up of cohorts such as the ET2DS will be important in determining what the impact of NAFLD is, particularly as mortality from cardiovascular disease is reduced. Such data will inform the decision whether to more comprehensively screen for NAFLD in the Type 2 diabetic population. Other important considerations are the development of an effective screening strategy and development of treatments that might slow or halt progression of NAFLD.

If screening were to be proposed then it is certainly possible to argue that all people with Type 2 diabetes should be screened, and the simple clinical scoring systems for fibrosis that have been outlined highlight the high-risk nature of this population. A more stratified approach, based on the data presented in the current work, might use markers such as BMI and glycaemic control to identify those at particularly high risk.

Currently there is no focused screening strategy designed to systematically diagnose NAFLD. Traditional LFTs are a poorly sensitive screening tool, but a high ALT is a strong indicator of liver pathology and should be further pursued. One approach would be to identify all those with hepatic steatosis on ultrasound as an initial step, and the current study would suggest that such screening can be carried out by an experienced sonographer as well as by radiologists. More challenging, however, is the identification of those with NASH or fibrosis (who are at highest risk of morbidity and mortality related to liver disease) as the available non-invasive tools, such as the ELF panel or cytokeratin-18, are expensive and not widely available clinically. It is to be hoped that an increasing body of evidence confirming a clinically important burden of liver disease will drive change in this area.

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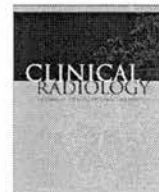
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## Appendix

### Publications

The following publications have been reproduced with the permission of the publishers and co-authors.

1. Williamson RM, Perry E, Glancy S et al. The use of ultrasound to diagnose hepatic steatosis in type 2 diabetes: intra- and interobserver variability and comparison with magnetic resonance spectroscopy. *Clinical Radiology* 2011; **66**: 434-439
2. Williamson RM, Price JF, Glancy S et al. Prevalence of and risk factors for hepatic steatosis and nonalcoholic fatty liver disease in people with Type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabetes Care* 2011; **34**: 1139-1144.  
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3. Williamson RM, Price JF, Hayes PC et al. Prevalence and markers of advanced liver disease in type 2 diabetes. *QJM* 2012; **105**: 425-432.



## Original Paper

# The use of ultrasound to diagnose hepatic steatosis in type 2 diabetes: Intra- and interobserver variability and comparison with magnetic resonance spectroscopy

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## ARTICLE INFORMATION

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**AIM:** To compare ultrasound gradings of steatosis with fat fraction (FF) on magnetic resonance spectroscopy (MRS; the non-invasive reference standard for quantification of hepatic steatosis), and evaluate inter- and intraobserver variability in the ultrasound gradings.

**MATERIALS AND METHODS:** Triple grading of hepatic ultrasound examination was performed by three independent graders on 131 people with type 2 diabetes. The stored images of 60 of these individuals were assessed twice by each grader on separate occasions. Fifty-eight patients were pre-selected on the basis of ultrasound grading (normal, indeterminate/mild steatosis, or severe steatosis) to undergo <sup>1</sup>H-MRS. The sensitivity and specificity of the ultrasound gradings were determined with reference to MRS data, using two cut-offs of FF to define steatosis,  $\geq 9\%$  and  $\geq 6.1\%$ .

**RESULTS:** Median (intraquartile range) MRS FF (%) in the participants graded on ultrasound as normal, indeterminate/mild steatosis, and severe steatosis were 4.2 (1.2–5.7), 4.1 (3.1–8.5) and 19.4 (12.9–27.5), respectively. Using a liver FF of  $\geq 6.1\%$  on MRS to denote hepatic steatosis, the unadjusted sensitivity and specificity of ultrasound gradings (severe versus other grades of steatosis) were 71 and 100%, respectively. Interobserver agreement within one grade was observed in 79% of cases. Exact intraobserver agreement ranged from 62 to 87%.

**CONCLUSION:** Hepatic ultrasound provided a good measure of the presence of significant hepatic steatosis with good intra- and interobserver agreement. The grading of a mildly steatotic liver was less secure and, in particular, there was considerable overlap in hepatic FF with those who had a normal liver on ultrasound.

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## Introduction

Non-alcoholic fatty liver disease (NAFLD), i.e., hepatic steatosis in the absence of a secondary cause, is a common cause of chronic liver disease. It is more common in people with type 2 diabetes than in the general population, and studies have suggested that it may affect up to 75% of individuals in this group.<sup>1–3</sup> It is important to detect this condition as it can result in considerable morbidity and mortality,<sup>4–7</sup> and may independently predict the risk of cardiovascular events.<sup>8</sup> Its identification can be used to initiate screening and target treatment for hepatic cirrhosis.

Ultrasound imaging is an established imaging modality in the diagnosis of hepatic steatosis, both clinically and in large-scale studies. Although several grading systems have been proposed for the assessment of hepatic steatosis using ultrasound, no consensus has been achieved.<sup>3,9–12</sup> Detection of hepatic fat is based on well-established characteristics including an echogenic parenchyma (especially in relation to the right kidney), posterior attenuation, and areas of focal fatty sparing.<sup>3,11–14</sup> Compared with liver biopsy, it has been reported to have a sensitivity of 83–100% and specificity of 84–100% in the diagnosis of hepatic steatosis.<sup>9,11,13,15</sup> It also has the advantages over biopsy of safety, ease of acquisition, and ability to assess the whole liver.

Although a degree of subjectivity is recognized when diagnosing hepatic steatosis by ultrasound examination, few studies have examined intra- and interobserver variability in making this diagnosis. One study of 25 patients with biopsy-proven NAFLD (less than a quarter of whom had diabetes) reported that the interobserver correlation between two radiologists in determining severity of steatosis was 0.40 (representing fair agreement), and the intraobserver correlation was 0.63 (suggesting more substantial agreement).<sup>9</sup> A different study comprising 168 patients who had undergone abdominal ultrasonography for a variety of abdominal disorders, demonstrated interobserver agreement between three radiologists regarding the presence and severity of hepatic steatosis of 0.40–0.51 and intraobserver agreement of 0.58 (moderate agreement).<sup>16</sup> In both studies, the assessment of the severity of steatosis was based on the presence and severity of increased echogenicity of the hepatic parenchyma and attenuation of the ultrasound beam.

In comparison with ultrasound imaging, magnetic resonance spectroscopy (MRS), the non-invasive reference standard for measurement of hepatic fat, is expensive but has a high correlation with liver fat concentration on biopsy, of the order of 0.7–0.9.<sup>17,18</sup> Different “normal” values for hepatic fat fraction (FF) on magnetic resonance imaging have been reported previously.<sup>19–21</sup> The Dallas Heart Study used MRS to determine hepatic FF in a population of 345 participants who had normal body mass index (BMI), glucose tolerance, and liver function tests, and non-excessive alcohol use (i.e., a very low risk for developing hepatic steatosis). In this group the 95th percentile of hepatic triglyceride content (expressed as grams of triglyceride per 100 g wet liver tissue) was 5.5%, corresponding to an MRS fat fraction of 6.1%,<sup>17</sup> and

this was taken as the upper limit of normal.<sup>20</sup> In an earlier smaller study, 28 healthy volunteers underwent magnetic resonance imaging (MRI) using a modified Dixon gradient echo technique. All had a magnetic resonance hepatic FF under 9% and this was later considered as being the upper limit of normal.<sup>19,21</sup> In the same study seven patients with cystic fibrosis underwent both MRI and liver biopsy: two patients who had no steatosis on biopsy had a FF on MRI of <9%; five patients who had mild to severe steatosis had a FF of >9%. A further study found that the geometric mean intrahepatic lipid content (percentage ratio of CH<sub>2</sub> lipid peak area relative to the water peak area) in 23 healthy volunteers to be 2.7 (range 0.2–77.4).<sup>22</sup> There is a paucity of data comparing qualitative ultrasound gradings with FF on MRS.

The aim of the present study was to compare ultrasound gradings of hepatic steatosis, performed by three graders, with hepatic FF on MRS, and also to examine inter- and intraobserver variability in these ultrasound gradings.

## Materials and methods

The selection of participants for the Edinburgh Type 2 Diabetes Study has been previously described in detail. Briefly, patients recorded as having type 2 diabetes, aged 60–74 years, were selected at random into sex and 5 year age bands from the Lothian Diabetes Register, a computerized database that contains details of over 20,000 patients with type 2 diabetes living in Lothian, Scotland. Invitations to participate in an initial clinic were sent to 5454 people and, of these, 1077 (19%) attended a baseline clinic (for a study investigating cognitive function). Of these, 939 participants re-attended for liver assessment by liver ultrasound examination (ultrasound<sub>1</sub>) at a specially set up research clinic. Ethical approval for the study had been gained from the local ethics committee and written informed consent was given by each participant. All ultrasound examinations were performed after a 4 h fast by a single ultrasonographer (grader 1), using a Sonoline Elegra Ultrasound Imaging System (Siemens Medical Systems, Washington, USA), software version 6, with a 3.5 MHz transducer. The appropriate pre-set (“medium” or “large” abdomen) was chosen for each subject according to the ultrasonographer’s judgement — these pre-sets remained constant throughout the study. Participants were scanned raised at an angle of 30–40° onto their left side. Right lobe images were subcostal or intercostal depending on the body habitus of the participant; left lobe images were obtained in the midline of the abdomen. The following images were obtained: longitudinal images of the right lobe and caudate lobe with inferior vena cava, right lobe with the portal vein, right lobe in the mid-clavicular line, right lobe of liver with kidney (at least two images) for comparison, right lobe close to the diaphragm and left lobe in its midline; transverse images of the right lobe at the level of the hepatic veins entering the inferior vena cava and at the level of the main portal vein, the right lobe close to the diaphragm, the right lobe at the level of the right portal vein and /or right hepatic vein, the right lobe of liver with kidney for comparison and

the left lobe at the level of the left hepatic vein and/or left portal vein. The liver was graded for evidence of hepatic steatosis using accepted criteria including a bright hepatic echo pattern (compared with the right kidney), increased attenuation of the echo beam (impaired visualization of the diaphragm or intrahepatic vessels) and the presence of focal fatty sparing.<sup>3,9–12</sup> Participants were given an overall liver grading based on a subjective measurement of the severity of steatosis: normal; indeterminate (i.e., possible slight increase in echogenicity or slightly impaired visualization of the diaphragm or intrahepatic vessels, or difficulty in grading as a result of a diseased or absent right kidney); mild steatosis (i.e., definite increase in echogenicity and/or definite impaired visualization of the intrahepatic vessels and diaphragm, no or little evidence of focal fatty sparing); severe steatosis (i.e., marked increase in echogenicity and/or poor or no visualization of the diaphragm and intrahepatic vessels, with or without focal fatty sparing).

### *MRS subgroup*

A subgroup of participants from the cohort described above were selected to undergo MRS. Twenty participants were selected at random into each of three groups on the basis of their ultrasound<sub>1</sub> grading: normal (with normal biochemical tests of liver function); indeterminate or mild steatosis (a pre-determined merging of these two gradings into one group); and severe steatosis. In addition, those with steatosis were selected following exclusion of a secondary cause (excess alcohol intake, hepatotoxic medication use, viral hepatitis, or autoimmune hepatitis) and, therefore, had NAFLD. Other exclusion criteria were maximal diameter over 60 cm (in order to fit the dimensions of the scanner) and contraindications to MRI. One participant who did not tolerate the procedure was replaced by another participant who had the same grading classification. MRS measurements were unsuccessful in two participants and, therefore, 58 participants who attended for imaging had successful FF measurements.

At the MRS visit, participants underwent a repeat ultrasound examination (ultrasound<sub>2</sub>), using the above protocol, 2 h prior to MRS. This measurement was made to ensure that the ultrasound grading reflected the presence or absence of steatosis in the liver at the time of MRS.

The MRS examination was performed using a 1.5 T HDX scanner (GE Healthcare, Amersham, UK) and consisted of a PRESS-localized volume of interest (VOI) of dimensions 30 × 30 × 30 mm, placed away from vascular structures. The system's body coil was used for transmission and reception. A repetition time (TR) of 5 s allowed substantial T1 relaxation of the signals, and a short (40 ms) echo time (TE) was used to minimize T2 decay. Automatic shimming achieved a water line width of typically 11 Hz. Sixteen acquisitions were averaged. Water-suppressed spectra (64 acquisitions) were also collected but are not reported here. The MRS data files were transferred from the scanner to a computer workstation for analysis. Quantification consisted of normalization for scanner transmit and receive gains and actual VOI volume, followed by modelling of the water and

fat peaks using the MRUI package ([www.mrui.uab.es](http://www.mrui.uab.es)). FF was defined as the ratio of the area under the fat peak to the combined areas under the water and fat peaks.

### *Intra- and interobserver variability*

Sixty participants from the MRS subgroup had ultrasound<sub>2</sub>, and 71 participants from the initial cohort had ultrasound<sub>1</sub>, graded by three independent graders using the four-point grading system outlined above (normal, indeterminate, mild steatosis, and severe steatosis). The 71 participants from the initial cohort represented all who were scanned during a particular calendar month. The three graders comprised the sonographer who performed the examinations (grader 1, 15 years experience), a consultant radiologist (grader 2, 12 years experience), and a medical trainee in radiology (grader 3, 2.5 years experience). Grader 1 scored images at the time of acquisition; still images from the examinations were recorded and saved for examination by graders 2 and 3. The three graders were blinded to each others' gradings and to the MRS result. All graders were unaware of the clinical and laboratory results of the participants.

After an interval of at least 2 months, each of the three graders re graded the still images from the 60 ultrasound<sub>2</sub> examinations, in a blinded fashion, to allow assessment of intraobserver variability.

### *Statistical analysis*

The median and interquartile ranges of FF on MRS were calculated in each group according to ultrasound gradings. The Kruskal–Wallis test was used to compare FF between all three groups, and the Mann–Whitney test was used for post-hoc comparisons between pairs of groups. Sensitivity and specificity of the ultrasound<sub>2</sub> gradings were determined with reference to MRS data, using two separate cut-offs of FF to define "normality", 9% and 6.1%.<sup>17,20,21</sup> The results for grader 1 were later adjusted to take into account the proportion of participants in the entire cohort receiving each grading. Inter- and intraobserver variability were analysed using the marginal homogeneity test. Data were analysed using SPSS version 14.0.

## **Results**

### *MRS subgroup*

All 58 participants with spectroscopy measurements underwent the repeat ultrasound examination (ultrasound<sub>2</sub>) and this was graded by three graders; the number of participants put into each category for each grader is listed in Table 1. For grader 1, median MRS FF was 4.2% (IQ range 1.2–5.7%) in the group graded normal, 4.1% (IQ range 3.1–8.5%) in the group graded indeterminate/mildly steatotic, and 19.4% (IQ range 12.9–27.5%) in the group graded severely steatotic (Fig 1). There was a significant difference in median FF between the three groups ( $p < 0.001$ ). The group with severe steatosis had significantly higher FF than either the normal ( $p < 0.001$ ) or the indeterminate/mild



**Table 1**

Fat fraction on magnetic resonance spectroscopy for each of three groups of gradings on ultrasound (normal, indeterminate/mildly steatotic, severely steatotic) as performed by three graders.

	Normal	Indeterminate/mild	Severe
Grader 1	4.2 (1.2–5.7) [n = 17]	4.1 (3.1–8.5) [n = 19]	19.4 (12.9–27.5) <sup>a</sup> [n = 22]
Grader 2	4.2 (1.3–5.7) [n = 16]	4.1 (2.6–7.9) [n = 20]	19.4 (12.9–27.5) <sup>a</sup> [n = 22]
Grader 3	4.2 (1.8–6.3) [n = 26]	6.0 (3.0–12.5) [n = 14]	20.1 (12.9–28.4) <sup>a</sup> [n = 18]

Data are median (interquartile range) [number of participants].

<sup>a</sup> Significant difference in the fat fraction between the normal, indeterminate/mild, and severe groups ( $p < 0.001$ ).

steatosis groups ( $p < 0.001$ ). There was no significant difference in FF between the normal and indeterminate/mild steatosis groups. Results were similar for the gradings of the other two graders (Table 1).

For grader 1, all 17 participants in the group who were graded as normal had a FF less than 9%, and 14 of these participants had a FF less than 6.1%. In the indeterminate/mildly steatotic ultrasound group, 16 participants had a FF less than 9% and, of these participants, 10 had a FF under 6.1%. In the severely steatotic group, all but one participant had a FF of greater than 9%, with the remaining participant having a FF of 8.45%. Results for the other two graders were similar and are listed in Table 2.

Sensitivity and specificity were calculated with both MRS FF cut-offs and results, with 95% confidence intervals, are presented in Table 3. For grader 1 using a liver FF of  $\geq 9\%$  on MRS to denote hepatic steatosis, the sensitivity of ultrasound in detecting indeterminate/mild/severe steatosis was 100% and specificity was 50%. If those graded indeterminate/mild steatosis were instead grouped with those graded normal (in view of the similar median MRS FF in these groups) sensitivity was 87.5% and specificity 97.1%. When a liver FF of  $\geq 6.1\%$  was used to define hepatic steatosis, the sensitivity of ultrasound in detecting indeterminate/mild/

severe steatosis was 90.3% and specificity was 51.9%. When those with indeterminate/mild steatosis were grouped with those graded normal, the sensitivity was 71% and specificity 100%. Results were extremely similar for grader 2. For grader 3, the sensitivity in detecting indeterminate/mild/severe steatosis was lower (87% and 78.1% for the 9% and 6.1% cut-offs, respectively) but specificity was higher (65.7 and 57.6% for the 9% and 6.1% cut-offs, respectively).

As the sensitivity and specificity of a test can be affected by the prevalence of the underlying condition (and in the present study, participants were selected according to presence of the condition on ultrasound, thereby pre-determining the prevalence), the results of grader 1 were adjusted to take account of the proportion of participants in the entire cohort of 939 participants who received each grading (24% normal, 19% indeterminate/mild steatosis, 57% severe steatosis). The results were not substantially different from the unadjusted data (Table 3).

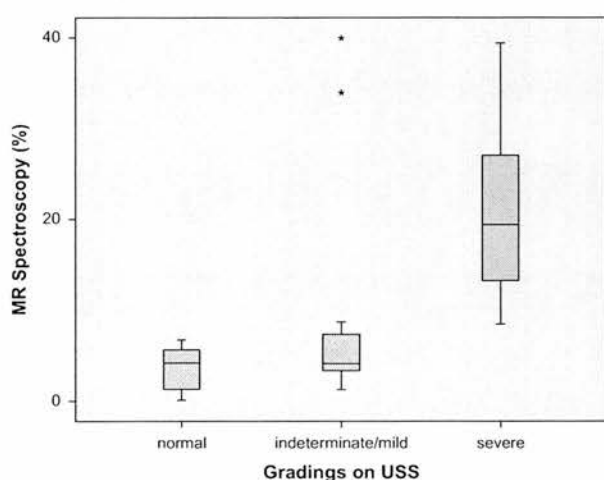
#### Inter- and intraobserver variability

Calculation of interobserver agreement between the three graders revealed exact consensus in 51% (67/131 given the same grade). Interobserver agreement within one grade was seen in 79% of cases (104/131). Interobserver variation of two grades was seen in 17% (22/131), and variation of three grades was seen in 4% (5/131). On pairwise analysis, there was no significant variation in the gradings of graders 1 and 2 (68% exact consensus), or between those of graders 2 and 3 (65% exact consensus). When the gradings of graders 1 and 3 were compared, however, there was found to be significant variation (disparity in 41% of results,  $p = 0.024$ ). When the 71 participants from the original cohort were examined separately, there was no significant variation between gradings from different graders. In contrast, there was significant variation between the gradings of the 60 participants in the MRS subgroup between graders 1 and 3 ( $p = 0.005$ ), and graders 2 and 3 ( $p = 0.011$ ).

Intraobserver variation was minimal for graders 1 and 2 (87 and 77% exact concordance between the first and second readings, respectively), but statistically significant for grader 3, with 38% disparity between readings, ( $p = 0.039$ ).

## Discussion

To the authors' knowledge this is the first study to examine both the relationship between ultrasound gradings of steatosis and MRS, and the inter- and intraobserver



**Figure 1** Boxplot showing median percentage fat on MRS in each of the three groups of ultrasound gradings by grader 1 (normal, indeterminate/mild steatosis, severe steatosis). Upper and lower ends of rectangles correspond to 75th and 25th centiles, respectively. Whiskers represent maximum and minimum values. Outliers in the indeterminate/mild steatosis group are marked by an asterisk. The group with severe steatosis had a significantly higher median FF than those with normal or indeterminate/mild steatosis ( $p < 0.001$ ).

**Table 2**Comparison of ultrasound gradings with proposed cut-offs of "normality" (<6.1% and 9%) of the fat fraction on magnetic resonance spectroscopy.<sup>19,20</sup>

MRS cut-off		Grading								
		Normal			Indeterminate/mild			Severe		
		Grader 1 (n = 17)	Grader 2 (n = 16)	Grader 3 (n = 26)	Grader 1 (n = 19)	Grader 2 (n = 20)	Grader 3 (n = 14)	Grader 1 (n = 22)	Grader 2 (n = 22)	Grader 3 (n = 18)
9%	< 9%	17	16	23	16	17	10	1	2	2
	≥ 9%	0	0	3	3	3	4	21	20	16
6.1%	< 6.1%	14	14	19	13	13	7	0	0	1
	≥ 6.1%	3	2	7	6	7	7	22	22	18

agreement in ultrasound grading. The present study was broad in its scope, including both participants who were likely to have a normal liver and those considered to have varying degrees of hepatic steatosis, mainly NAFLD. In addition, the use of ultrasound has been examined in diagnosing hepatic steatosis in people with type 2 diabetes, a population who are at high risk of developing hepatic steatosis and in whom screening for the condition should be considered.

When compared to MRS, a normal ultrasound grading was an excellent predictor of a normal hepatic FF, and a severe steatosis grading was an excellent predictor of the presence of hepatic steatosis. The grading of a mildly steatotic liver was less reliable, and in particular, considerable overlap in hepatic FF was evident compared with those who had a normal liver on ultrasound. Therefore, it is reasonable to suppose that those graded indeterminate/mild steatosis had subtle changes on ultrasound that were within the limits of normality. In clinical practice, a grading of indeterminate/mild steatosis should be interpreted in conjunction with clinical and biochemical factors, and it may be appropriate to repeat the ultrasound examination after an interval of time. The performance of the three graders was similar when gradings were compared to MRS. It should be emphasized that ultrasound images were obtained within hours of MRS measurements to ensure that they were truly comparable, as hepatic steatosis can vary over time.

Individuals were pre-selected into the MRS subgroup based on their ultrasound<sub>1</sub> gradings, with the intention of having broadly equal numbers of participants in each of three groups. It was acknowledged that the proportion in each group was different to the proportion of each grade in

the entire cohort, as graded by grader 1. Therefore, adjusted figures were provided for comparison, with the assumption that if the number of participants in each ultrasound grading group had been selected according to overall proportion in the total cohort, then the proportion of participants in each group testing below and above the cut-offs of MRS FF would have stayed the same.

No accepted definition of "normal" FF on MRS currently exists. Biopsy data from normal livers and MRS FF have rarely been compared. Furthermore, studies investigating "healthy" volunteers may be skewed by the presence of overweight individuals or those who drink alcohol in amounts greater than 14 units/week — both of which are known to predispose to hepatic steatosis. It seems likely that the proposed figure of 6.1% (corresponding to 5.5% hepatic triglyceride content, if expressed as grams of triglyceride per 100 g wet liver tissue) for the upper limit of normal is the most accurate available, as this was calculated using a population from which those with risk factors for steatosis, such as BMI >25 kg/m<sup>2</sup>, glucose intolerance, and excessive alcohol use had been excluded.<sup>20</sup>

It is acknowledged that MRS gives information regarding FF within a portion of the liver rather than in the liver as a whole, and that it was theoretically possible that participants with focal fatty change could be misclassified as a result. In this study, however, the volume of interest for MRS was fairly large in order to minimize any effects of local heterogeneity. Indeed, examination of MRS data from the 15 participants with focal fatty sparing on ultrasound revealed FFs that were in keeping with their overall ultrasound grading (data not shown).

**Table 3**

Sensitivity and specificity of ultrasound in identifying presence of hepatic steatosis, using two cut-offs of hepatic fat fraction (&lt;9% and &lt;6.1%) on magnetic resonance spectroscopy.

Cut-off	Sensitivity (unadjusted)	Specificity (unadjusted)	Sensitivity (adjusted)	Specificity (adjusted)
<b>9% Cut-off</b>				
Normal versus indet/mild/severe steatosis	100 (82.8–100)	50 (32.8–67.2)	100 (87–100)	56 (35.2–75)
Normal/indet/mild steatosis versus severe steatosis	87.5 (66.5–96.7)	97.1 (82.9–99.8)	93.9 (78.4–99)	92 (72.4–98.6)
<b>6.1% Cut-off</b>				
Normal versus indet/mild/severe steatosis	90.3 (73.1–97.5)	51.9 (32.4–70.8)	94.7 (80.9–99.1)	60 (36.4–80)
Normal/indet/mild steatosis versus severe steatosis	71 (51.8–85.1)	100 (84.5–100)	86.8 (71.1–95.1)	100 (80–100)

Data are shown for the grouping of the indeterminate/mild steatosis group with either the normal or the severe steatosis groups. Both unadjusted values and those that have been adjusted to take account of the proportion of each grading in the entire study cohort are presented. Ultrasound gradings are from grader 1. Data are presented as % (95% confidence interval). Indet, indeterminate.

Comparison of the gradings by three experienced graders revealed some variation, but exact concordance in a majority of cases, with most differences being small (one-point only); intraobserver concordance was high in two of three graders, but was lower for the grader with the least experience (a medical trainee in radiology). Of the two previous studies to examine inter- and intraobserver variability, one was a small study whose scope was limited to patients with abnormal biochemical liver function tests and biopsy-proven NAFLD.<sup>9</sup> The other was more comparable in size and scope to the present study, but results cannot be directly compared due to different statistical methods used. The present study compared gradings from graders with different training (a sonographer, a senior consultant radiologist, and a medical trainee in radiology). This reflects clinical practice, and is particularly relevant at a time when increasing numbers of ultrasound examinations are being performed by experienced sonographers.

Participants in the MRS substudy were necessarily less than 60 cm in maximal diameter so excluding people with very severe obesity. It is widely acknowledged that ultrasound is more difficult in the most obese patients, and it could be anticipated that accuracy (compared to MRS) and inter-/intraobserver concordance would be less in this population. It is, however, important to note that interobserver variation in the MRS subgroup was not superior to that of the essentially randomly selected group from this general study population.

In conclusion, hepatic ultrasound provides a good measure of the presence of hepatic steatosis, and in particular, a normal grading and severe steatosis grading were excellent predictors of a normal and high MRS FF, respectively. Hepatic ultrasound has advantages over other measures of hepatic fat in its ease of use and lack of adverse side-effects, and is likely to remain a mainstay of investigation, both in research and clinical situations.

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# Prevalence of and Risk Factors for Hepatic Steatosis and Nonalcoholic Fatty Liver Disease in People With Type 2 Diabetes: the Edinburgh Type 2 Diabetes Study

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**OBJECTIVE**—Type 2 diabetes is an established risk factor for development of hepatic steatosis and nonalcoholic fatty liver disease (NAFLD). We aimed to determine the prevalence and clinical correlates of these conditions in a large cohort of people with type 2 diabetes.

**RESEARCH DESIGN AND METHODS**—A total of 939 participants, aged 61–76 years, from the Edinburgh Type 2 Diabetes Study (ET2DS)—a large, randomly selected population of people with type 2 diabetes—underwent liver ultrasonography. Ultrasound gradings of steatosis were compared with magnetic resonance spectroscopy in a subgroup. NAFLD was defined as hepatic steatosis in the absence of a secondary cause (screened by questionnaire assessing alcohol and hepatotoxic medication use, plasma hepatitis serology, autoantibodies and ferritin, and record linkage to determine prior diagnoses of liver disease). Binary logistic regression was used to analyze independent associations of characteristics with NAFLD.

**RESULTS**—Hepatic steatosis was present in 56.9% of participants. After excluding those with a secondary cause for steatosis, the prevalence of NAFLD in the study population was 42.6%. Independent predictors of NAFLD were BMI, lesser duration of diabetes, HbA<sub>1c</sub>, triglycerides, and metformin use. These remained unchanged after exclusion of participants with evidence of hepatic fibrosis from the group with no hepatic steatosis.

**CONCLUSIONS**—Prevalences of hepatic steatosis and NAFLD were high in this unselected population of older people with type 2 diabetes, but lower than in studies in which ultrasound gradings were not compared with a gold standard. Associations with features of the metabolic syndrome could be used to target screening for this condition.

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**N**onalcoholic fatty liver disease (NAFLD), defined as hepatic steatosis in the absence of a secondary cause, is the commonest cause of liver disease in westernized countries, affecting up to

33% of the general population (1,2) and up to 75% in some subgroups such as obese patients (3). An association between NAFLD and type 2 diabetes is well established. Diabetes has been

shown to be a risk factor for the development of NAFLD and its progression to more advanced liver disease including fibrosis, cirrhosis, and hepatocellular carcinoma (4,5). Furthermore, data suggest that some classes of antidiabetic agents (6,7) may be used as treatments in NAFLD.

Despite the recognized association between type 2 diabetes and NAFLD, few large-scale studies of the prevalence of NAFLD in unselected populations of people with type 2 diabetes are available. Studies estimating the prevalence using liver ultrasound have been performed in secondary care rather than community setting and have generally examined small numbers of patients (8). A large study in Italian outpatients with type 2 diabetes reported that 85% had hepatic steatosis and that 70% met the criteria for NAFLD (defined in that study as hepatic steatosis without evidence of excess alcohol consumption, viral hepatitis, or causative medications) (9). This study, however, was confined to patients attending a hospital clinic and also failed to systematically identify and exclude participants with less common causes of liver disease such as autoimmune hepatitis. Furthermore, ultrasound measurements were not compared with a gold standard in the population studied—an important factor given the variable sensitivity and specificity of ultrasound assessment for hepatic steatosis (10).

Given the rising incidence and prevalence of type 2 diabetes, an accurate estimate of the prevalence of NAFLD, as well as its clinical correlates, is important to predict the number of patients who will require to be monitored for more advanced liver disease or who may benefit from future disease-modifying agents. The aim of the current study was to determine the prevalence of NAFLD in a large, randomly selected population of older people with type 2 diabetes, using magnetic resonance spectroscopy (MRS)

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to confirm ultrasound grading classifications and thorough screening for secondary causes of liver disease, and to examine the correlation of NAFLD with clinical and biochemical characteristics.

## RESEARCH DESIGN AND METHODS

The recruitment and baseline examination of subjects for the Edinburgh Type 2 Diabetes Study (ET2DS) have been described previously (11). Briefly, subjects recorded as having type 2 diabetes and aged 60–74 years were selected at random by sex and 5-year age bands in years 2006–2007 from the Lothian Diabetes Register. This is a comprehensive database of people with type 2 diabetes living in Lothian, a region in the south-east of Scotland that includes the city of Edinburgh and its surrounding towns and countryside, including both patients attending a hospital clinic and those managed solely in primary care. Study participants ( $n = 1,066$ ) have been shown previously to be representative, in terms of age, HbA<sub>1c</sub>, duration of diabetes, insulin treatment, and total cholesterol, of all those randomly selected to participate ( $n = 5,454$ ) and therefore of the target population of older men and women with type 2 diabetes living in the general population (12). At the time of recruitment into the study, participants attended a baseline examination that included measurement of demographic and anthropometric variables (age, sex, BMI, and waist circumference), plasma HbA<sub>1c</sub>, total cholesterol, platelets, and hyaluronic acid. One year after baseline examination, 1,054 participants were invited to attend a year 1 clinic for assessment of liver structure and function. A total of 12 participants were not invited due to withdrawal ( $n = 2$ ), refusal of consent for contact ( $n = 5$ ), unsuitable for contact ( $n = 3$ ), or death ( $n = 2$ ). A total of 939 subjects (88% of the original cohort) participated in the year 1 clinic. Of those subjects invited but who did not attend the year 1 clinics ( $n = 114$ ), 19 were uncontactable, 61 unable or unwilling to attend, 21 repeatedly cancelled or failed to attend appointments, and 13 had died.

The ET2DS was approved by the local research ethics committee, and all participants gave written informed consent for baseline and year 1 examinations.

### Ultrasound examination and comparison with MRS

Subjects attended the year 1 research clinic after a 4-h fast for an ultrasound

examination of abdomen. All ultrasound examinations were performed by a single ultrasonographer, who was unaware of the clinical and laboratory results of the participants, using a Sonoline Elegra Ultrasound Imaging System (Siemens Medical Systems, Issaquah, WA), software version 6, with a 3.5-MHz transducer. The liver was graded for markers of hepatic steatosis using established criteria (13–15) including a bright hepatic echo pattern (compared with the echo response of the right kidney), increased attenuation of the echo beam, and the presence of focal fatty sparing. Participants were given an overall liver grading based on a subjective measurement of the severity of steatosis: grade 0, normal appearance of liver on ultrasound and initially graded as a “normal ultrasound”; grade 1, possible slight increase in echogenicity or slightly impaired visualization of the diaphragm or intrahepatic vessels, or difficulty in grading as a result of a diseased or absent right kidney—initially termed an “indeterminate ultrasound”; grade 2, definite increase in echogenicity and/or definite impaired visualization of the intrahepatic vessels and diaphragm, no or little evidence of focal fatty sparing, initially graded as “evidence of mild steatosis on ultrasound”; grade 3, marked increase in echogenicity and/or poor or no visualization of the diaphragm and intrahepatic vessels, with or without focal fatty sparing, initially graded as “evidence of severe steatosis on ultrasound.” Evidence of hepatic cirrhosis was also sought systematically.

Comparison of the ultrasound gradings for hepatic steatosis with <sup>1</sup>H MRS, the noninvasive gold standard for quantification of hepatic fat, in a subgroup of 58 participants has previously been described in detail (16). In brief, 17 participants with a grade 0 ultrasound grading, 19 with grade 1 or 2, and 22 with grade 3 underwent MRS. A grade 0 grading on ultrasound was associated with a “normal” hepatic fat fraction on MRS of <6.1% (17,18) in 14 of 17 cases (and a fat fraction of <9% [19,20] in all cases) and was accepted as “no steatosis.” A grading of grade 3 on ultrasound was associated with and MRS hepatic fat fraction  $\geq 6.1\%$  in all cases, and this was therefore taken as “definite steatosis.” The group with grade 1 or 2 on ultrasound had an MRS fat fraction of <6.1% in 13 of 19 cases, and a fat fraction of <9% in 16 of 19 cases, thus displaying considerable overlap with those regarded as

normal. In view of this, the group with grade 1 or 2 on ultrasound scan was considered to have a “probable normal” scan. If a liver FF of  $\geq 6.1\%$  on MRS was used to denote hepatic steatosis, sensitivity, specificity, and positive and negative predictive values (adjusted for the portion of the whole study cohort receiving each grading) of ultrasound in detecting “definite steatosis” were 86.8%, 100%, 100%, and 80.0%, respectively.

### Clinical examination

Average alcohol intake per week over the previous year, a history of alcohol excess, and use of hepatotoxic medications within the previous 6 months were determined by questionnaire. Average alcohol intake was determined using two questions adapted from the AUDIT-C screening tool (21): “How often did you have a drink containing alcohol in the past year? Consider a “drink” to be a can or a bottle of beer, a glass of wine, or one cocktail or a measure of spirits (like scotch, gin, or vodka)” (a drink was considered to be the equivalent of one unit of alcohol); and “How many drinks did you have on a typical day when you were drinking in the last year?”

Those participants with evidence of hepatic steatosis or abnormal blood tests of liver function had further investigations performed including serology for hepatitis B and C, antinuclear antibody (ANA), antismooth muscle antibody, antimitochondrial antibody, and ferritin. All participants had brachial blood pressure, serum triglycerides, and HDL cholesterol measured.

Information on previous diagnoses of chronic liver disease was collected from participants by questionnaire at year 1 and supplemented using data on liver diagnoses, which had been obtained at baseline via record linkage to hospital discharges at the Information and Services Division (ISD) of NHS Scotland.

### Definition of NAFLD

NAFLD was defined as the presence of definite hepatic steatosis on ultrasound scan (i.e., grade 3) in the absence of a secondary cause for hepatic steatosis. Secondary causes were defined as alcohol consumption  $\geq 14$  units/week (22) or participant report of current/previous alcohol excess; use of hepatotoxic medication (2) (glucocorticoids, isoniazid, methotrexate, amiodarone, and tamoxifen) within the 6 months prior to the year 1 clinic; positive hepatitis B or C

serology; ferritin concentration  $\geq 1,000$   $\mu\text{g/L}$  (milder hyperferritinemia can be associated with obesity, insulin resistance, and NAFLD [2,23]); clinically significant positive immunology titers (antismooth muscle antibody titer  $\geq 1:160$  [24] or antimitochondrial antibody titer  $\geq 1:40$  [25]); or a previous diagnosis of a persistent secondary cause for chronic liver disease. Subjects were considered to have a previous diagnosis of a secondary cause for chronic liver disease if ISD linkage revealed such a diagnosis, or if a participant report of a diagnosis was confirmed by their medical records. Subjects were excluded from calculations on the prevalence of NAFLD if data on the above measures were missing such that a secondary cause could not be excluded.

### Definition of metabolic syndrome

As per Adult Treatment Panel III criteria, subjects were considered to have the metabolic syndrome if, in addition to type 2 diabetes, they had at least two of the following: blood pressure  $\geq 130/85$  or on antihypertensive treatment; triglycerides  $\geq 1.7$  mmol/L or taking a fibrate; HDL cholesterol  $< 1.04$  mmol/L (men) or  $1.29$  mmol/L (women); waist circumference  $> 102$  cm (men) or  $88$  cm (women).

### Statistical analysis

Analysis was performed using SPSS software version 14.0. Data that did not conform to the normal distribution (duration of diabetes and triglycerides) were log-transformed prior to parametric analysis. Statistical analysis included the independent *t* test and  $\chi^2$  tests to compare characteristics between groups. Binary logistic regression analysis was used to assess the independence of variables in their association with hepatic steatosis and NAFLD. Variables included in this model were age, sex, BMI, duration of diabetes, HbA<sub>1c</sub>, systolic blood pressure, HDL and LDL cholesterol, triglycerides, and the use of metformin, sulfonylureas, thiazolidinediones, incretin mimetics, and insulin. Alcohol use  $\geq 14$  units/week or previous alcohol misuse was used as a categorical variable in the model examining hepatic steatosis, but not that examining NAFLD. Results were reported as estimated odds ratios (ORs) and 95% CI.

## RESULTS

### Subject characteristics

The characteristics of 939 participants (aged 61–76 years) attending the year 1

clinic are shown in Table 1. Baseline characteristics of subjects attending the year 1 clinical examination were similar to those of the total ET2DS population suggesting that the study population attending for liver assessment remained representative of the target general population with type 2 diabetes.

### Prevalence of hepatic steatosis and NAFLD

Of the 939 subjects undergoing abdominal ultrasound, data on steatosis grading was incomplete for 2 subjects, and these were excluded from the analysis. Hepatic steatosis was graded as definite (grade 3) on ultrasound examination in 56.9% ( $n = 533$ ) of the remaining participants. The appearance of the liver was normal (grade 0) in 23.5% ( $n = 220$ ) of participants. Twenty-two subjects (2.3%) were graded as grade 1 (in three of these cases, it was commented that the liver was difficult to scan as it was located high up under the costal margin) and a further 16.9% ( $n = 158$ ) were graded as grade 2, giving a total of 180 subjects (19.2%) who were classified as “probable normal.” In addition, 0.4% subjects ( $n = 4$ ) had a liver in which ultrasound identified cirrhosis—steatosis gradings in these subjects were grade 0 in two participants, grade 1 in one participant, and grade 2 in one participant.

Among those with evidence of definite hepatic steatosis (grade 3), 123 subjects had evidence of one or more secondary causes for steatosis: 78 had alcohol intake  $\geq 14$  units/week or a history of excess; 40 had used a hepatotoxic medication, as outlined above, in the previous 6 months; 1 each had serological evidence of hepatitis B and C; 3 had ferritin levels  $\geq 1,000$   $\mu\text{g/L}$ ; 1 had antismooth muscle antibody titer  $\geq 1:160$ ; 1 had antimitochondrial antibody titer  $\geq 1:40$ ; and 7 had previously diagnosed liver disease (2 had alcoholic liver disease, 1 hemochromatosis, 1 autoimmune hepatitis, 1 recurrent cholangitis, 1 biliary cirrhosis, and 1 hepatic carcinoid metastases). In a further 19 subjects data were missing such that a secondary cause could not be excluded. In addition, a further 160 participants had at least one positive immunology titer  $\geq 1:40$ , which was not considered significant. Of these, most had borderline ANA or antismooth muscle titers ( $\leq 1:80$ ;  $n = 120$ ); 40 participants had ANA titer  $\geq 1:160$ .

Using the predefined criteria, 391 out of 918 participants with a full dataset had definite hepatic steatosis on ultrasound grading with no secondary cause, giving a prevalence of NAFLD of 42.6% in this study population.

Table 1—Characteristics of subjects at year 1 clinic

Characteristic	Year 1
<i>n</i>	939
Age (years)	68.9 $\pm$ 4.2
Sex, % ( <i>n</i> ) male	52.0 (488)
Race, % ( <i>n</i> ) Caucasian	98.3 (923)
BMI measured at baseline clinic ( $\text{kg/m}^2$ )	31.3 $\pm$ 5.7
Waist circumference measured at baseline clinic (cm)	106.7 $\pm$ 12.8
Duration of diabetes (years)	9.0 $\pm$ 6.4
HbA <sub>1c</sub> (%)	7.19 $\pm$ 1.06
Diet controlled, % ( <i>n</i> )	19.4 (182)
Oral antidiabetic agent users, % ( <i>n</i> )	74.4 (699)
Insulin users, % ( <i>n</i> )	15.8 (148)
Systolic blood pressure (mmHg)	138.1 $\pm$ 18.5
Diastolic blood pressure (mmHg)	74.1 $\pm$ 9.6
Total cholesterol (mmol/L)	4.15 $\pm$ 0.81
HDL cholesterol (mmol/L)	1.23 $\pm$ 0.34
LDL cholesterol (mmol/L)	2.17 $\pm$ 0.68
Triglyceride (mmol/L)	1.66 $\pm$ 0.90
Statin users, % ( <i>n</i> )	81.6 (767)
Aspirin users, % ( <i>n</i> )	67.7 (636)
ACE inhibitor users, % ( <i>n</i> )	52.0 (488)
Current or ex-smokers, % ( <i>n</i> )	60.0 (563)

Data are mean  $\pm$  SD or proportions in whole cohort of participants unless otherwise indicated.

### Clinical and biochemical associations with hepatic steatosis and NAFLD

Physical and biochemical characteristics of study participants according to steatosis groups are shown in Table 2. In univariate analysis, participants in the group with definite steatosis (grade 3) were significantly younger and had lesser duration of diabetes than the combined normal/probably normal groups (grades 0, 1, and 2) ( $P < 0.05$ ). BMI, waist circumference, HbA<sub>1c</sub>, diastolic blood pressure, triglycerides, and prevalence of alcohol intake over 14 units/week were significantly higher, and HDL cholesterol significantly lower, in the definite steatosis group. Metformin use was more common in the definite steatosis group. No significant differences in the use of other antidiabetic agents (sulfonylureas, glitazones, incretin mimetics, or insulin) were found.

In multivariate analysis, independent predictors of definite hepatic steatosis (compared with those classed as normal/probable normal) on ultrasound scan were BMI (OR 1.07 [95% CI 1.04–1.10]), lesser duration of diabetes (OR for log duration diabetes 0.53 [0.30–0.92]), HbA<sub>1c</sub> (OR 1.35 [1.15–1.59]), triglycerides (OR for log triglycerides 20.76 [0.85–50.85]), alcohol intake  $\geq 14$  units/week (OR 3.13 [1.80–5.43]), and use of metformin (OR 2.19 [1.59–3.00]).

Similar results were obtained when those with secondary causes for liver

disease were excluded from analysis. Independent predictors of NAFLD were BMI (OR 1.07 [95% CI 1.03–1.10]), lesser duration of diabetes (OR for log duration diabetes 0.46 [0.25–0.83]), HbA<sub>1c</sub> (OR 1.29 [1.08–1.54]), triglycerides (OR for log triglycerides 17.57 [6.68–46.18]), and use of metformin (OR 2.25 [1.60–3.17]).

Of the 400 participants who had gradings of normal/probable normal liver parenchyma on ultrasound scan, 74 had at least one of the following features suggestive of possible hepatic fibrosis or cirrhosis: spleen size  $\geq 13$  cm (16 participants); hyaluronic acid  $>75$  ng/mL (the upper end of normal on the laboratory reference range) in the absence of joint disease (54 participants); or platelet count  $<150 \times 10^9/L$  (20 participants). If these participants were excluded from regression analysis, independent predictors of definite steatosis and NAFLD were unchanged (data not shown).

**CONCLUSIONS**—This is the first study to examine the prevalence of hepatic steatosis and NAFLD in a population of people with type 2 diabetes using an ultrasound classification that has been refined by comparison with MRS and with detailed exclusion of secondary causes of steatosis. The study population included the full clinical spectrum of type 2 diabetes and included people who were being managed in the community as well as in hospital clinics.

Previous studies have estimated that a hepatic fat fraction on MRS of under 6.1–9% is consistent with a normal liver, whereas hepatic steatosis is associated with higher fat fractions (17–20). Comparison of our ultrasound gradings with MRS suggested that the “definite steatosis” grading was an excellent predictor for the presence of hepatic steatosis (16). In contrast, considerable overlap was observed between those graded initially as having “indeterminate” or “mild” steatosis (grade 1 or 2) and the normal group, and it was considered that these participants probably had a normal liver. Our prevalence of hepatic steatosis, at 56.9%, was therefore considerably lower than in previous studies including that of a large Italian population of patients with type 2 diabetes, in which 85.3% were considered to have hepatic steatosis (9). In view of these findings, caution should be used with regard to the prevalences reported by previous studies that have used ultrasound measurements that have been less rigorously corroborated for a diagnosis of hepatic steatosis.

NAFLD was the most common cause for steatosis, accounting for 76% of all cases. In comparison with previous studies, our analysis had the advantage of a more systematic identification and exclusion of secondary causes of liver disease in those with steatosis, both by the use of ISD linkage to identify previously diagnosed chronic liver disease and by the routine measurement of autoantibody

Table 2—Comparison of participant characteristics across gradings of steatosis

Characteristic	Steatosis grade			
	Grade 0 (n = 220)	Grade 1 (n = 22)	Grade 2 (n = 158)	Grade 3 (n = 533)
Age (years)	69.4 $\pm$ 4.2	68.5 $\pm$ 4.8	69.5 $\pm$ 4.4	68.5 $\pm$ 4.0*
Sex, % (n) male	59.9 (132)	40.9 (9)	49.4 (78)	50.1 (267)
BMI measured at baseline clinic (kg/m <sup>2</sup> )	28.8 $\pm$ 5.18	35.1 $\pm$ 8.4	30.7 $\pm$ 4.9	32.4 $\pm$ 5.5*
Waist circumference (cm)	102.4 $\pm$ 13.5	112.6 $\pm$ 18.7	104.7 $\pm$ 11.6	108.9 $\pm$ 12.0*
Duration of diabetes (years)	9.6 $\pm$ 7.6	10.5 $\pm$ 6.0	9.8 $\pm$ 6.5	8.4 $\pm$ 5.8*
HbA <sub>1c</sub> (%)	7.02 $\pm$ 0.97	7.15 $\pm$ 0.93	6.99 $\pm$ 0.98	7.33 $\pm$ 1.12*
Systolic blood pressure (mmHg)	140.1 $\pm$ 21.5	141.2 $\pm$ 22.4	135.9 $\pm$ 18.6	137.9 $\pm$ 16.7
Diastolic blood pressure (mmHg)	73.4 $\pm$ 9.5	76.3 $\pm$ 11.2	72.1 $\pm$ 9.5	74.9 $\pm$ 9.4*
Total cholesterol (mmol/L)	4.14 $\pm$ 0.81	4.53 $\pm$ 0.81	4.01 $\pm$ 0.75	4.17 $\pm$ 0.82
HDL cholesterol (mmol/L)	1.28 $\pm$ 0.69	1.16 $\pm$ 0.27	1.30 $\pm$ 0.35	1.19 $\pm$ 0.32*
LDL cholesterol (mmol/L)	2.24 $\pm$ 0.70	2.57 $\pm$ 0.72	2.08 $\pm$ 0.65	2.14 $\pm$ 0.67
Triglyceride (mmol/L)	1.37 $\pm$ 0.69	1.76 $\pm$ 0.67	1.37 $\pm$ 0.63	1.86 $\pm$ 1.00*
Metabolic syndrome, % (n) present	70.2 (153)	86.4 (19)	78.3 (123)	91.2 (485)*
Alcohol intake, % (n) over 14 units/week	6.4 (14)	0.0 (0)	7.6 (12)	12.9 (69)*
Metformin use, % (n)	48.2 (106)	50.0 (11)	63.3 (100)	70.9 (378)*

Data are mean  $\pm$  SD unless otherwise indicated. \*Significant difference grade 3 vs. grade 0/1/2 by *t* test or  $\chi^2$  test,  $P < 0.05$ .

titors and ferritin in all participants with steatosis. The use of these additional measures within the group with steatosis identified four patients with likely hemochromatosis, two with likely autoimmune hepatitis, two with primary biliary cirrhosis, one with recurrent cholangitis, and one with hepatic carcinoma metastases; these accounted for 1.9% of this group. The systemic identification and exclusion of subjects with any possible secondary cause of steatosis from the diagnosis of NAFLD may have caused an underestimation of the prevalence of NAFLD, which may in some cases have been a coexistent pathology. It is recognized that the cutoffs used to define exclusions to the diagnosis of NAFLD are, to some extent, arbitrary and the prevalence will depend upon the precise definition used.

The majority of exclusions from the diagnosis of NAFLD were on the basis of excess alcohol intake. It is recognized that estimation of alcohol consumption can be unreliable, and in particular that subjects and investigators may underestimate intake. This, in turn, can lead to overestimation of the prevalence of NAFLD. The prevalence of alcohol intake  $\geq 14$  units/week as a secondary cause for steatosis was relatively low in our study population (14.6%) when compared with a similar age band in the general population of England, in which the prevalence of alcohol intake  $\geq 21$  units/week for men and  $\geq 14$  units/week for women has been quoted as 23% and 10%, respectively (26). While this may represent an underestimation of intake, it is also possible that it reflects genuinely lower alcohol consumption in a frailer population in which use of multiple prescription medications is not uncommon.

Subjects in this study were all aged 61–76 years at the time of examination and were predominantly Caucasian. Previous studies have shown that the prevalence of NAFLD increases with age (although we did not find evidence of that within our age range), and therefore it is possible that the prevalence of NAFLD in our study population was greater than that of the general diabetic population. The results may be less applicable to populations in other countries, particularly those in which the prevalence of other causes of liver disease (such as alcoholic liver disease and viral hepatitis) is markedly different to that in the U.K.

It has previously been shown that NAFLD is associated with features of the

metabolic syndrome within the general population, and the same was true in this population of people with type 2 diabetes. The association of a shorter duration of diabetes with liver disease has been described before. One possible explanation is that the greater degree of hyperinsulinemia in early type 2 diabetes drives uptake of free fatty acids by hepatocytes. Unexpectedly, the use of metformin was associated with the presence of NAFLD, independently of BMI and glycemic control. Previous studies of the use of metformin in NAFLD have shown a positive or neutral effect in individuals (6), and it therefore seems unlikely that there is a causative link between metformin use and NAFLD here. It is possible that those participants who were on metformin had other risk factors for NAFLD that were not accounted for in the present analysis, such as inflammatory markers. Furthermore, it is possible that results were confounded by indication, i.e., participants may have been previously prescribed metformin in an attempt to treat hepatic steatosis. Finally, it is possible that the significant association between metformin and steatosis was a consequence of a type 1 statistical error.

It is acknowledged that while data on most variables were gathered concomitantly with hepatic ultrasound examination, BMI was calculated 1 year previously during the baseline examination. It is therefore possible that the results of the regression analysis are less robust than they would have been with fully contemporaneous data collection. However, changes in BMI over 1 year would in most cases have been small and randomly distributed throughout the study population, and this should not have substantially affected the results of analysis.

Another limitation of this study is that subjects did not have a liver biopsy and histological examination, the gold standard technique for identifying steatosis; performance of this invasive procedure would have been neither feasible nor ethical in a population study of this magnitude. It was recognized that some participants with a normal ultrasound scan could have undiagnosed hepatic fibrosis and thus be at the severe end of the spectrum of NAFLD. Of note, any misclassified cases would tend to underestimate the prevalence of NAFLD and reduce rather than magnify any differences in clinical associations between groups. Furthermore, reanalysis excluding those participants with a normal liver

ultrasound scan but other evidence of possible fibrosis revealed similar results to the main analysis. A further limitation is that only approximately one-fifth of patients invited to attend the baseline clinic from the Lothian Diabetes Register did so. Significantly, however, analysis revealed that this population was representative of that invited in terms of duration of diabetes, HbA<sub>1c</sub>, and treatment with insulin.

In conclusion, in assessing the prevalence of NAFLD in people with type 2 diabetes, this study has advantages of robust ultrasound gradings, systematic exclusion of other causes for liver disease, and a relatively large, unselected population of older individuals. In the future, it is possible that the association of liver disease with other features of the metabolic syndrome could be used to target screening for NAFLD. Further research is required to ascertain whether the risk of progression of NAFLD to cirrhosis in patients with type 2 diabetes in the clinical setting is as high as that predicted—if so, this would provide further impetus toward early diagnosis so that they might be eligible for entry into clinical trials, screening for complications, and ultimately new therapies.

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R.M.W. designed the study, collected and analyzed data, and wrote the manuscript. J.F.P. designed the study, analyzed data, and edited the manuscript. S.G. designed the study, collected data, and edited the manuscript. E.P. designed the study, collected data, and edited the manuscript. L.D.N. collected data and edited the manuscript. P.C.H. designed



the study and edited the manuscript. B.M.F. edited the manuscript. L.A.F.V.L. collected data and edited the manuscript. G.I.J. designed the study and edited the manuscript. R.M.R. designed the study and edited the manuscript. M.W.J.S. was principal investigator, designed the study, analyzed data, and edited the manuscript.

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Research clinics were performed in the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh, U.K., and the MRS was carried out in the Scottish Funding Council Brain Imaging Centre, Western General Hospital, Edinburgh, U.K.

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## Prevalence and markers of advanced liver disease in type 2 diabetes

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### Summary

**Background:** Type 2 diabetes is a risk factor for progression of non-alcoholic fatty liver disease (NAFLD) to fibrosis and cirrhosis. We examined the prevalence of advanced liver disease in people with type 2 diabetes and analysed the effectiveness of liver function tests (LFTs) as a screening tool.

**Methods:** Participants ( $n=939$ , aged 61–76 years) from the Edinburgh Type 2 Diabetes Study, a randomly selected population of people with type 2 diabetes, underwent abdominal ultrasonography. Hyaluronic acid (HA) and platelet count/spleen diameter ratio (PSR) were used as non-invasive markers of hepatic fibrosis and portal hypertension. Subjects were screened for secondary causes of liver disease that excluded them from a diagnosis of NAFLD. The efficacy of LFTs [alanine

aminotransferase (ALT) and gamma-glutamyltransferase (GGT)] in screening for liver disease was determined.

**Results:** Cirrhosis was identified by ultrasound in four participants (0.4%). Ten (1.1%) had evidence of portal hypertension (PSR < 909), and two (0.2%) had hepatocellular carcinoma. Fifty-three participants (5.7%) had evidence of hepatic fibrosis (HA > 100 ng/ml in the absence of joint disease); a further 169 had HA > 50 ng/ml. In participants with NAFLD-related fibrosis (HA > 100 ng/ml), 12.5% had an elevated ALT level and 17.5% had an elevated GGT level.

**Conclusions:** The prevalence of hepatic fibrosis and cirrhosis were lower than expected. The use of LFTs to screen for liver disease missed most cases of fibrosis predicted by raised HA levels.

### Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to hepatic steatosis in the absence of a defined secondary cause. NAFLD is associated with type 2 diabetes and estimates of prevalence of NAFLD

in this group have varied, the highest being 70%.<sup>1</sup> Using a validated ultrasound measure and detailed exclusion of secondary causes of liver disease, we recently reported a prevalence of NAFLD of 42.6% in over 900 older people with type 2 diabetes.<sup>2</sup>

NAFLD encompasses a spectrum of liver disease from simple steatosis to steatohepatitis, fibrosis and ultimately cirrhosis. In the general population of people with simple steatosis secondary to NAFLD, an estimated 8% progress to advanced fibrosis and cirrhosis, and this figure is higher when steatohepatitis is evident at presentation.<sup>3</sup> Although type 2 diabetes is considered a risk factor for the progression of NAFLD,<sup>4,5</sup> few studies have examined the prevalence of advanced liver disease in this population. Existing evidence is either indirect or has originated from small studies or those undertaken in highly selected populations. In one population-based study, the risk of death from liver cirrhosis was 2.5 times higher in people with type 2 diabetes compared with the general population.<sup>6</sup> In another study of 132 patients with NAFLD (44 of whom had diabetes) who had undergone liver biopsy in a tertiary referral centre, 25% of those with diabetes had cirrhosis, compared to 10% of the non-diabetic cohort.<sup>7</sup>

Screening for liver disease in Type 2 diabetes generally involves the measurement of standard liver function tests (LFTs) alone. Previous studies, however, suggest that LFTs may be poorly sensitive in differentiating patients with steatosis and NAFLD from those with normal livers.<sup>8,9</sup> Furthermore, it is known that NAFLD can progress to fibrosis without significant elevation of LFTs.<sup>5,9</sup> It has been suggested that lowering the upper limit of normal of alanine aminotransferase (ALT) to 30 U/l in men and 19 U/l in women may improve sensitivity of this measure in detecting NAFLD,<sup>10</sup> but this has not been widely adopted. It is therefore likely that many cases of NAFLD in the people with diabetes are not identified and progressive liver disease is often missed. However, few data are available on the applicability of LFTs to diagnose hepatic fibrosis in people with diabetes.

Other approaches have been taken in the pursuit of a reliable non-invasive method of distinguishing those patients who have significant fibrosis. Hyaluronic acid (HA) is a component of the extracellular matrix, whose production is increased, and degradation by the liver decreased, in hepatic fibrosis. Several small studies have suggested that cut-off values of 42–50 ng/ml give optimal sensitivity and specificity in determining the presence of significant fibrosis.<sup>11–14</sup> Higher levels may be associated with increasing severity of liver disease and increased specificity in diagnosing fibrosis and cirrhosis. One study suggested that a level >100 ng/ml had a 78% specificity and 83% sensitivity for predicting cirrhosis, with specificity being raised to 96% if a cut-off of 300 ng/ml was used.<sup>15</sup> One potential confounder is

that levels of HA can also be raised in patients with active joint disease.

Other routinely available markers that are predictive of fibrosis have been combined into scoring systems. The BAAT score assigned one point for each of increased age, BMI, ALT and triglycerides, and while a score of two gave 71% sensitivity and 80% specificity in detecting septal fibrosis, a score of three gave 14% sensitivity but 100% specificity.<sup>16</sup> The BARD score allocated one point for each of increased BMI and the presence of diabetes, and two points for aspartate aminotransferase (AST):ALT ratio >0.8 – with a cut-off of two points, the positive predictive value (PPV) and negative predictive value (NPV) of determining severe fibrosis were 43% and 96%, respectively.<sup>17</sup> The NAFLD fibrosis score, using a formula combining age, BMI, the presence of diabetes, AST:ALT ratio, albumin and platelet levels, classified 75% of participants low- or high-risk groups for severe fibrosis, with a low cut-off (below –1.455) giving a NPV of 93% and a high cut-off (above 0.676) a PPV of 90%.<sup>18</sup>

Ultrasound scanning has diagnostic accuracy of 80–86% in the diagnosis of cirrhosis or extensive fibrosis in chronic liver disease<sup>19</sup> and can detect consequences of portal hypertension such as increased spleen size. A low platelet count is a well-established marker of splenomegaly, and the platelet count/spleen diameter ratio (PSR) has been shown to be a reliable predictor of oesophageal varices in people with cirrhosis<sup>20</sup> and can therefore be used as a surrogate marker of portal hypertension.

Hepatocellular carcinoma (HCC) is a well-recognized complication of hepatic cirrhosis. Diabetes is an established risk factor for HCC and in one population-based study, diabetes was associated with around a 2.5-fold increase in incidence of HCC,<sup>4</sup> independent of alcohol intake and viral hepatitis. The prevalence of HCC in the general population of people with type 2 diabetes, however, remains unclear.

The present study aimed to estimate the prevalence of fibrosis, cirrhosis and HCC, using a range of different markers and scoring systems, in a randomly selected population of people with type 2 diabetes [the Edinburgh Type 2 Diabetes Study (ET2DS)] and to analyse the effectiveness of LFTs in screening for liver disease in this population.

## Methods

### Participants

The selection of subjects for the ET2DS has been described previously in detail.<sup>2,21</sup> Briefly, subjects

recorded as having type 2 diabetes mellitus, aged 60–74 years, were selected at random into sex and 5-year age bands from the Lothian Diabetes Register, a computerized database, which contains details of >20 000 patients with type 2 diabetes living in Lothian, Scotland, and includes both patients attending hospital and those seen only in primary care. Invitations to participate were sent to 5454 people and, of these, 1066 (20%) attended a baseline clinic. The clinical characteristics upon which the diagnosis of type 2 diabetes mellitus was confirmed has previously been described in detail.<sup>21</sup> In brief, a diagnosis of diabetes was accepted in any individual treated with oral antidiabetic agents and/or insulin, and in any individual treated with dietary modification alone whose HbA1c was >6.5%. The clinical records of all subjects treated with dietary modification alone and with an HbA1c  $\leq$  6.5% at the research clinic were reviewed by a consultant diabetologist (MWJS) to ensure that the diagnosis of diabetes was accurate. With regard to the classification of 'type 2' diabetes, the clinical records of individuals who either (i) started on insulin within one year of diagnosis of diabetes, (ii) reported evidence of pancreatic surgery or disease at the research clinic or (iii) were treated with insulin and were aged <35 years at diagnosis were also reviewed. Such individuals were considered to be at greatest risk of mis-classification. Any subject in whom it was not possible to confirm a clinical diagnosis of type 2 diabetes by review of hospital or general practitioner records was excluded. Study participants have been shown previously to be representative of all those randomly selected to participate and therefore of the target population of older men and women with type 2 diabetes living in the general population.<sup>2</sup> A total of 939 subjects participated in the Year 1 clinic, a re-attendance rate of 88%. Baseline characteristics of those attending the Year 1 clinic have been shown to be similar to the full study population, suggesting that they remained representative of the target population.<sup>2</sup> All participants gave written informed consent. The local ethics committee gave ethical approval for the study.

## Procedures

Subjects attended a specially established research clinic, based in the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh, at baseline and after one year (Year 1). At Year 1, participants attended, after a 4-h fast, for an ultrasound examination of abdomen and venous blood sampling.

All ultrasound examinations were performed by a single ultrasonographer as previously described.<sup>2,21</sup> Participants were given an overall liver grading based on a subjective measurement of the severity of steatosis. Validation of the ultrasound gradings for hepatic steatosis with <sup>1</sup>H magnetic resonance spectroscopy, the non-invasive gold standard for quantification of hepatic fat, in a subgroup of 58 participants has previously been described in detail.<sup>22</sup> Evidence of cirrhosis was sought systemically and spleen length was measured.

Alcohol intake, use of hepatotoxic medications (amiodarone, isoniazid, methotrexate, tamoxifen and glucocorticoids) within the previous 6 months and history of joint disease were determined by questionnaire. Those participants with evidence of hepatic steatosis or abnormal blood tests of liver function had further tests performed for liver disease performed including serology for Hepatitis B and C, anti-nuclear antibody (ANA), anti-smooth muscle antibody, anti-mitochondrial antibody, ferritin and alpha fetoprotein (AFP). In addition, all participants (including those with normal liver and LFTs) seen after a certain date had these same screening blood tests performed ( $n=644$ ). Triglycerides and HDL cholesterol were measured at Year 1. BMI, platelets and HA had been previously measured at the baseline clinic.

Participants who had significant abnormalities on hepatic USS or blood tests were referred for standard follow-up within the National Health Service. Those with focal lesions on USS had these reviewed by a consultant radiologist and received further imaging as necessary, and those with raised AFP > 20 kU/l were referred for review in a liver clinic.

Data on previous diagnoses of chronic liver disease were collected from participants by questionnaire at Year 1. In addition, data on liver diagnoses were obtained at baseline from discharge summaries via record linkage at the Information and Services Division of NHS Scotland (ISD linkage).

Upper limits of normal on the laboratory reference range of bilirubin (Bi), ALT, AST and gamma-glutamyl transferase (GGT) were 18  $\mu$ mol/l, 50 U/l, 45 U/l and 55 U/l respectively. Analysis was also carried out using lower upper limits of normal for ALT—30 U/l for men and 19 U/l for women.<sup>10</sup>

## Definition of NAFLD, PSR, hepatic fibrosis and HCC

NAFLD was defined as the presence of hepatic steatosis on ultrasound scan in the absence of a secondary cause for hepatic steatosis. In addition, for this study, the definition was widened to include people with markers of fibrosis but no evidence of hepatic

steatosis on ultrasound, in acknowledgement of the fact that steatosis can regress in patients with significant fibrosis. Secondary causes were defined as alcohol consumption  $\geq 14$  U/week or participant report of a current or previous problem with alcohol excess, use of hepatotoxic medication (glucocorticoids, isoniazid, methotrexate, amiodarone and tamoxifen) within the 6 months prior to the Year 1 clinic, positive hepatitis B or C serology, ferritin  $> 1000$   $\mu\text{g/l}$ , clinically significant positive immunology titres (anti-smooth muscle antibody titre  $\geq 1:160$  or anti-mitochondrial antibody titre  $\geq 1:40$ ) or a previous diagnosis of a secondary cause for chronic liver disease (alcoholic liver disease, autoimmune hepatitis, primary biliary cirrhosis, cholangitis or liver metastases).<sup>2</sup> Subjects were considered to have a previous diagnosis of a secondary cause for chronic liver disease if ISD linkage revealed such a diagnosis, or if a participant report of a diagnosis was confirmed by their medical records.

PCR was defined as the ratio of platelet count (per cubic millimetre) to spleen diameter (millimetre).<sup>20</sup>

Hepatic fibrosis was defined on the basis of HA levels—values of  $>100$  ng/ml, in the absence of arthritis, were taken as evidence of definite fibrosis and possible cirrhosis.<sup>15</sup> A cut-off of 50 ng/ml was taken to indicate possible fibrosis;<sup>11–14</sup> values  $>75$  ng/ml are also reported as this is the upper limit of normal on our laboratory reference range.

HCC was defined, in accordance with accepted guidelines,<sup>23</sup> by one of the following: two imaging techniques showing a focal lesion over 2 cm in diameter with arterial hypervascularisation; one imaging technique showing a focal lesion  $>2$  cm in diameter in association with AFP levels  $>400$  ng/ml (484 kU/l); suggestive histology following biopsy of a lesion.

### Fibrosis clinical scoring systems

Participant scores on established scoring systems (BAAT, BARD and NAFLD fibrosis scores) were calculated. The BAAT<sup>16</sup> and BARD<sup>17</sup> scoring systems are outlined above. NAFLD fibrosis score was calculated as previously published.<sup>18</sup> Scores of 2–4 on both the BAAT and BARD scores were considered to be predictive of hepatic fibrosis. On the NAFLD fibrosis score, a score of  $>0.676$  was considered to be predictive of fibrosis, and a score of  $-1.455$ – $0.676$  was indeterminate for fibrosis. In view of the fact that these systems have been developed to assess the severity of NAFLD alone, they were used only in those participants in whom another secondary cause for liver disease had been excluded.

### Statistical analyses

Statistical analysis was performed using SPSS software version 14.0 (SPSS Inc., Illinois, USA). Data not conforming to a normal distribution (in this study GGT measurements) were log-transformed prior to parametric analysis. Statistical analysis included the one-way analysis of variance and chi-squared test to compare biochemical characteristics between groups. PPV and NPV were used to analyse the utility of LFTs in predicting hepatic fibrosis.

### Results

#### Prevalence of markers of fibrosis, cirrhosis and HCC

Baseline characteristics of the 939 participants, aged 61–75 years, who attended the Year 1 clinic are shown in Table 1. Hepatic cirrhosis on ultrasound scan was found in four members of the study population, a prevalence of 0.4%. Portal hypertension,

**Table 1** Characteristics of subjects at Year 1 clinic

Characteristic	Year 1 (n = 939)
Age (years)	68.9 $\pm$ 4.2
Sex [% (n) male]	52.0 (488)
Race [% (n) Caucasian]	98.3 (923)
BMI- measured at baseline clinic (kg/m <sup>2</sup> )	31.3 $\pm$ 5.7
Duration of diabetes (years)	9.0 $\pm$ 6.4
HbA1c (%)	7.2 $\pm$ 1.1
Diet-controlled [% (n)]	19.4 (182)
Oral anti-diabetic agent users [% (n)]	74.4 (699)
Metformin users [% (n)]	63.7 (598)
Thiazolidinedione users [% (n)]	17.5 (164)
Insulin users [% (n)]	15.8 (148)
Systolic blood pressure (mmHg)	138.1 $\pm$ 18.5
Diastolic blood pressure (mmHg)	74.1 $\pm$ 9.6
Total cholesterol (mmol/l)	4.2 $\pm$ 0.8
High-density lipoprotein cholesterol (mmol/l)	1.2 $\pm$ 0.3
Low-density lipoprotein cholesterol (mmol/l)	2.2 $\pm$ 0.7
Triglyceride (mmol/l)	1.7 $\pm$ 0.9
Statin users [% (n)]	81.6 (767)
Aspirin users [% (n)]	67.7 (636)
Angiotensin converting enzyme inhibitor users [% (n)]	52.0 (488)
Current or ex-smokers [% (n)]	60.0 (563)

Data are mean  $\pm$  standard deviation or proportions, in whole cohort of participants unless stated (number of participants in brackets)

defined as a platelet count/spleen diameter ratio <909, was present in 10 participants (1.1%), including one participant who had cirrhosis on ultrasound scan. A total of 876 participants were fully assessed for secondary causes of liver disease. Of these, 663 participants had no secondary cause identified, and in this subgroup findings suggestive of fibrosis or cirrhosis would be presumed secondary to NAFLD. Hepatic cirrhosis on ultrasound scan was found in one person (0.2%) in this subgroup and low platelet count/spleen diameter in four other people (0.6%).

The prevalence of definite hepatic fibrosis, defined as HA level >100 ng/ml in the absence of joint disease, was 5.7% in the entire study population of 939 participants. The corresponding figures for HA level >50 ng/ml and >75 ng/ml were 23.6% and 12.2%, respectively. Of the population, 0.5% had HA level >300 ng/ml. An additional 20.6% of participants had HA levels >50 ng/ml but also had evidence of arthritis. Of the 13 participants with evidence of cirrhosis on ultrasound scan or a low platelet count/spleen diameter ratio, four (30.8%) had HA levels 50–100 ng/ml and a further six (46.2%) had HA levels >100 ng/ml.

In the subgroup of participants with no secondary cause for liver disease, 40 participants (6.1%) had HA >100 ng/ml in the absence of joint disease, 166 participants (25.2%) had HA >50 ng/ml, 86 participants (13.0%) had HA >75 ng/ml and 4 participants (0.6%) had HA >300 ng/ml.

Two participants had definite HCC, a prevalence in the study population of 0.2%. In one case, this was confirmed on histology following biopsy of a 3.7 cm liver mass on a background of cirrhosis seen on USS, in association with raised AFP levels of 40 kU/l. A second participant had a very high AFP

(2712 kU/l); no mass was seen on USS, but appearances on magnetic resonance imaging were supportive of a diagnosis of HCC. Neither participant had a secondary cause for liver disease, giving a prevalence of 0.3% in the group with possible NAFLD.

### Comparison of clinical fibrosis scoring systems with radiological and biochemical markers

The three clinical scoring systems for prediction of fibrosis secondary to NAFLD were used as alternative means of calculating prevalence of fibrosis in the subgroup with no secondary cause for liver disease. The BARD score was positive in 92.6% compared to 79.3% for the BAAT score, while 16.4% of the subgroup had a positive NAFLD Fibrosis Score, and in a further 66.8% this score was indeterminate.

The only participant in this subgroup to have cirrhosis on ultrasound scan had positive BARD, BAAT and NAFLD Fibrosis scores. When the prevalence of positive fibrosis scores were compared across three groups based on HA measurements [HA <50 ng/ml (or arthritis present); HA 50–100 ng/ml in the absence of arthritis; and HA >100 ng/ml in the absence of arthritis], the only positive correlation was with the NAFLD fibrosis score with the prevalence of a positive score rising from 13.7% to 21.3% to 42.5% in the three HA groups respectively ( $P < 0.001$ ). Conversely, 72.5% of those with a positive NAFLD fibrosis score had a HA measurement >50 ng/ml.

### Comparison of tests of liver function with markers of fibrosis

Standard tests of liver function were compared between three groups according to the HA level in

**Table 2** Comparison of standard tests of liver function between participants with no secondary cause for liver disease, grouped according to severity of liver disease

	HA <50 ng/ml (n=494)	HA 50–100 ng/ml (n=126)	HA >100 ng/ml (n=40)	Cirrhosis on USS (n=1)
ALT(U/l)	33.3 (12.5)	34.0 (13.5)	34.9 (14.5)	38.0
ALT >50 U/l, n (%)	34 (6.9)	9 (7.1)	5 (12.5)	0 (0)
AST (U/l)	30.1 (9.9)	31.5 (9.4)	35.3 (12.6)*	62.0
AST >45 U/l, n (%)	29 (5.9)	8 (6.3)	4 (10.0)	1 (100)
GGT (U/l, mean logGGT)	1.2 (0.3)	1.2 (0.3)	1.4 (0.5)*	1.6
GGT >55 U/l, n (%)	36 (7.3)	7 (5.6)	7 (17.5)*	0 (0)

HA, hyaluronic acid.

\*Significant differences between the groups stratified according to HA level by one-way ANOVA or chi-squared test ( $P < 0.05$ ).

Data are mean  $\pm$  standard deviation unless stated otherwise.

**Table 3** Positive and negative predictive values of standard LFTs [ALT (using standard and alternative upper limits of normal) and GGT] in the diagnosis of fibrosis secondary to NAFLD, as defined using HA levels or the NAFLD Fibrosis Score

	ALT (cut-off 50 U/l)		ALT (alternative cut-offs <sup>a</sup> )		GGT (cut-off 55 U/l)	
	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
HA <sup>b</sup> (cut-off 50 ng/ml)	29.2	75.1	25.1	74.8	28.0	75.1
HA <sup>b</sup> (cut-off 100 ng/ml)	10.4	94.3	6.0	93.7	14.0	94.6
NAFLD Fibrosis Score <sup>c</sup>	15.9	13.6	20.5	10.3	22.9	14.6

<sup>a</sup>Upper limit of normal taken as 30 U/l for men and 19 U/l for women.

<sup>b</sup>Participants with arthritis were grouped with those with a HA level below the cut-off

<sup>c</sup>PPVs are predictive of a NAFLD fibrosis score >0.676; NPVs are predictive of a NAFLD fibrosis score less than -1.455.

participants with no secondary cause for liver disease (Table 2). Mean levels of ALT, AST and GGT remained within the laboratory normal reference range in all three groups. Similarly, mean levels of ALT, AST and GGT remained within the normal range in all three groups when participants were divided according to NAFLD fibrosis score (less than -1.455; -1.455 to 0.676; and >0.676: data not shown). The PPV and NPV of ALT and GGT in predicting fibrosis as diagnosed using HA levels and the NAFLD fibrosis score are presented in Table 3.

The PPV and NPV of proposed new upper limits of normal of ALT (>30 U/l for men; >19 U/l for women) are also shown in Table 3.

## Conclusions

Detection of advanced liver disease using non-invasive methods is challenging. This study examines the prevalence of markers of hepatic fibrosis and cirrhosis, and their relationship to standard tests of liver function, in a large randomly selected cohort of older people with Type 2 diabetes. The prevalence of hepatic fibrosis was 5.7% and that of ultrasound-diagnosed cirrhosis under 1%. The use of LFTs as a screening tool missed the majority of cases of fibrosis predicted by raised HA levels.

Previous reports have estimated the prevalence of hepatic fibrosis, cirrhosis and HCC secondary to NAFLD in this group to be higher than in the non-diabetic population—but results have often either been extrapolated from other data or based on results from relatively highly selected patient populations. The present study has the advantage that participants were selected at random from a register of all people in the South-East of Scotland who had type 2 diabetes and therefore represented the entire spectrum of this condition. It is the first study to include people with diabetes who are

receiving their routine review in the community as well as those in the secondary care setting. It is possible that the high rate of use of anti-diabetic agents in the population, particularly metformin, may have had a disease modifying effect on NAFLD and contributed to the lower than expected rates of fibrosis and cirrhosis.

Subjects in this study were all aged 61–76 years at the time of examination and were predominantly Caucasian. Previous studies have shown that the prevalence of NAFLD and its progression to fibrosis and cirrhosis increases with age. It is therefore possible that the results from our study population may not be representative of those from other age ranges. At baseline, approximately one-fifth of people invited to attend from the Lothian Diabetes Register did so—analysis suggested that this population was representative of that invited, most importantly in terms of duration of diabetes, HbA1c and treatment with insulin.

HA was the main marker of fibrosis used in this study, with cut-offs for fibrosis and cirrhosis defined as in previous reports.<sup>11–15</sup> Liver biopsy, the gold standard, would have been neither feasible nor ethical, and in contrast HA measurement is relatively non-invasive. We attempted to improve specificity by excluding participants with arthritis (another cause of raised HA) from the fibrosis categories, and also by using a high cut-off (100 ng/ml) for our definition of definite evidence of fibrosis. While we can be reasonably confident that the proportion of the study population with fibrosis is >5.7% (those with HA levels >100 ng/ml, without joint disease), it is possible that the prevalence of fibrosis was lower than the 26% predicted by HA levels >50 ng/ml.

A low platelet count is a well-established consequence of hypersplenism, and the platelet count/spleen diameter has previously been validated as a marker of portal hypertension.<sup>20</sup> This validation was,

however, carried out in patients with known cirrhosis, and it is possible that its use is less applicable in our study population. In particular, it is possible an alternative diagnosis may have underpinned the finding of hypersplenism in some of our participants, and this may have contributed to the lack of overlap seen in our measures of cirrhosis and platelet count/spleen diameter ratio.

The prevalence of HCC in our cohort was 0.2%, a figure which is comparable to that previously found on screening both a general population in Italy (prevalence of 0.7%).<sup>24</sup> It is also in keeping with the only previous large-scale study, carried out in Taiwan,<sup>25</sup> that has looked at prevalence of HCC in a subgroup of people with type 2 diabetes, in which the prevalence was 0.1%. This population, unlike ours, had a high prevalence of viral hepatitis. In contrast, prevalence of HCC is higher in populations with known cirrhosis, and in one small-scale study the prevalence in an obese group with cryptogenic cirrhosis was 30%.<sup>26</sup>

Although significant increases were noted in the values of some conventional LFTs through the groups with increasing evidence of liver damage, mean values remained within normal limits. This is consistent with previous studies which have shown that progression to cirrhosis can occur without major perturbation in the LFT values.<sup>5</sup> Although the NPVs for ALT and GGT were high in predicting lower HA levels, these values are affected by the very low population prevalence of fibrosis. The use of lower values for the upper limit of normal of ALT did not materially change the results. Currently, liver screening in type 2 diabetes is carried out using conventional LFTs alone, and our data suggest that such an approach misses the majority of cases of hepatic fibrosis.

The only clinical scoring system to positively correlate with HA measurements was the NAFLD fibrosis score, which predicted definite fibrosis in 16.4% of participants with no secondary cause for liver disease. A further 66.8% of subjects had an indeterminate score. This is a much higher proportion than in the original article, which suggested that such patients should be considered for liver biopsy.<sup>18</sup> The BARD and BAAT scores both estimated that a very high percentage of participants in this study had fibrosis but in view of the emphasis on age, BMI and diabetes in these scoring systems, this could have been anticipated. Interestingly, when the BARD score was validated in a subgroup with type 2 diabetes in the initial study, the AUC was lower than in the group as a whole (0.53 vs. 0.80). This supports the concept that these more simple scoring systems, while useful in the general population of patients with NAFLD, may be less applicable to populations

of people with type 2 diabetes. The risk of their indiscriminate use is that they may identify large numbers of patients for biopsy without any definitive evidence of a high prevalence of advanced liver disease.

In conclusion, this study provides an estimate of prevalence of hepatic fibrosis in a large population of older people with type 2 diabetes. There are challenges in detecting advancing liver disease, and current screening methods using conventional LFTs are almost certainly inadequate. Further work is required to refine identification of these patients, particularly in those in whom liver biopsy would be difficult to justify. This may require a panel of biochemical markers, for example, the Enhanced Liver Fibrosis panel or cytokeratin-18, or a radiological method such as transient elastography.

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