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The epidemiology of chronic liver disease in older people with type 2 diabetes mellitus: the Edinburgh Type 2 Diabetes Study

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Abstract

Increasingly chronic liver disease is being acknowledged as a complication of type 2 diabetes, in particular non-alcoholic fatty liver and non-alcoholic fatty liver disease. Rates of non-alcoholic fatty liver are higher in people with type 2 diabetes than in the general population, with prevalence rates believed to be between 40-70%. Given the aging Scottish population and the obesity driven diabetes epidemic, the problem of chronic liver disease is likely to increase.

Despite this there has been little investigation into the natural history of non-alcoholic fatty liver disease and the risks of clinically significant chronic liver disease in community based cohorts because diagnosis has been heavily reliant on liver biopsy. The use of liver biopsy is limited in both research and clinical practice due to its associated high mortality (1/1000) and morbidity and also due to practical limitations (sampling variability, semi-quantitative scoring systems). As a result the use of non-invasive markers of liver injury (non-specific liver injury, steatosis, steatohepatitis, liver fibrosis and surrogates of advanced portal hypertension) are rising, in the diagnosis of chronic liver disease, however, their utility in both community cohorts and patients with type 2 diabetes has not been widely studied.

The aims of the studies presented in the thesis, using the Edinburgh Type 2 Diabetes Study, were: (i) to describe the distributions of a range of non-invasive markers of steatohepatitis and liver fibrosis in older people with type 2 diabetes, their relationship with metabolic and liver disease risk factors, and to compare the agreement of different non-invasive markers of hepatic fibrosis; (ii) to determine the frequency (prevalence and incidence) of and risk factors for clinically significant chronic liver disease in people with type 2 diabetes; and (iii) to determine the importance of chronic liver disease as a risk factor (or risk marker) for cardiovascular mortality or morbidity in type 2 diabetes.

Prior to undertaking this work I undertook a detailed systematic review of the literature relating to the use of non-invasive markers of hepatic fibrosis to inform the choice of markers used in the study.

Examination of a wide range of potential markers of steatohepatitis and liver fibrosis found varied relationships with diabetes history. Most commonly, elevated markers of steatohepatitis and liver fibrosis were associated with older age and higher body fat measures. However, most of these relationships between liver markers and body fat measures lost statistical significance when limiting the population to only those with hepatic steatosis and/or non-alcoholic fatty liver disease.

There were marked differences in the associations between different liver fibrosis markers and potential diabetes and metabolic risk factors, suggesting that these markers are not actually measuring the same underlying "fibrosis" condition. There was poor correlation between the five markers of liver fibrosis studied. Using the top vigintile (5%) of each marker resulted in excellent agreement on the absence of advanced liver disease but poor agreement on the presence of advanced liver disease.

The prevalence of clinically significant CLD (defined as cirrhosis, HCC or gastro-oesophageal varices) was 2.2% - 0.9% diagnosed prior to enrolment with an additional 1.4% identified by study investigations. Over nearly 6 years of follow-up, only 1.4% of the cohort developed incident clinically significant CLD.

Higher levels of systemic inflammation, steatohepatitis and hepatic fibrosis markers were associated with both unknown prevalent and incident clinically significant chronic liver disease. Less than half of participants developing incident significant disease were identified as high risk by the study investigations. Abnormal liver enzymes were statistically significantly associated with incident cases, however the presence of hepatic steatosis was not.

There were 372/1033 (36.0%) patients with prevalent CVD and 319 (30.9%) with prevalent CAD at baseline. After mean follow-up of 4.4 years there were 44/663 incident CVD events, including 27 CAD events. There were 30/82 CVD related deaths.

However, risk of dying from or developing CVD was no higher in subjects with steatosis than in those without. There was also no statistically significant relationship between CVD and steatohepatitis or liver fibrosis. The only statistically

significant relationship between CVD and any liver markers was with GGT (prevalent CVD, OR 1.28, p=0.007; incident CAD, OR 2.35, p=0.042), suggesting that in our study population, CLD may have little effect on the development of, or mortality from, CVD.

In conclusion, the potential for using non-invasive biomarkers to diagnose clinically significant chronic liver disease in type 2 diabetes remains limited, however chronic liver disease is a significant problem in older people with type 2 diabetes and is frequently undiagnosed.

Declaration

I declare that this thesis is of my own composition, and that the research contained within it is entirely my own unless stated otherwise. No part of the work has been submitted for any other degree or professional qualification.

Joanne R. Morling

Edinburgh, December 2014

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Relevant publications, presentations and awards

Publications

Morling JR, Fallowfield JA, Williamson RM, Robertson CM, Glancy S, Guha IN, Strachan MWJ, Price JF. γ-Glutamyltransferase, but not markers of hepatic fibrosis, is associated with cardiovascular disease in older people with type 2 diabetes mellitus: the Edinburgh Type 2 Diabetes Study. Diabetologia 2015 (in press).

Morling JR, Fallowfield JA, Guha IN, Nee LD, Glancy S, Williamson RM, Robertson RM, Strachan MWJ, Price JF. Using non-invasive markers to identify liver fibrosis in people with type 2 diabetes mellitus: the Edinburgh Type 2 Diabetes Study. Journal of Hepatology 2014;60(2):384-391.

Morling JR, Fallowfield JA, Williamson RM, Nee LD, Jackson AP, Glancy S, Reynolds RM, Hayes PC, Guha IN, Strachan MWJ, Price JF. Non-invasive hepatic markers (ELF and CK18) in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. Liver International 2014;34(8):1267-77.

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Morling JR, Fallowfield JA, Williamson RM, Robertson CM, Nee LD, Glancy S, Guha IN, Strachan MWJ, and Price JF. Cardiovascular disease and markers of liver injury in Type 2 diabetes: the Edinburgh Type 2 Diabetes Study (ET2DS). Diabetic Medicine 2014;31(s1):74 (poster).

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Morling JR, Williamson RM, Robertson CM, Fallowfield JA, Guha IN, Strachan MWJ and Price JF. Hepatic inflammation and fibrosis markers are associated with cardiovascular risk factors but not cardiovascular disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study (ET2DS). Diabetic Medicine 2013; 30(s1):157 (poster).

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Morling JR, Fallowfield JA, Guha IN, Williamson RM, Ali M, Nee LD, Glancy S, Strachan M and Price J. The prevalence and incidence of chronic liver disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. Association of British Clinical Diabetologists SpR Meeting 2014 (oral).

Morling JR, Fallowfield JA, Guha IN, Williamson RM, Ali M, Nee LD, Glancy S, Strachan M and Price J. The burden of chronic liver disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. Association of British Clinical Diabetologists Autumn Meeting 2014 (poster).

Morling JR, Strachan MWJ, Fallowfield J, Guha N, Williamson RM, and Price JF. Liver fibrosis in people with type 2 diabetes mellitus: The Edinburgh Type 2

Diabetes Study. MRS Clinical Scientists in Training Meeting, London February 2012 (poster).

Awards

Diabetes UK, Type 2 Diabetes Research Award, first runner up, March 2015

Association of British Clinical Diabetologists SpR prize winner, November 2014

Clinical Academic Training Fellowship, Diabetes UK, March 2010

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Abbreviations

ALD	alcoholic liver disease
ALT	alanine aminotransferase
AMA	anti-mitochondrial antibody
ANA	anti-nuclear antibody
APRI	Aspartate aminotransferase to Platelet Ratio Index
ARFI	acoustic radiation force impulse
ASMA	anti-smooth muscle antibody
AST	aspartate aminotransferase
AUROC	area under the receiver operating curve
BMI	body mass index
CAD	coronary artery disease
CDS	cirrhosis discriminant score
CI	confidence interval
CK18	cytokeratin-18
CLD	chronic liver disease
COL-IV	type IV collagen
CRP	C-reactive protein
CV	cardiovascular
CVD	cardiovascular disease
dBP	diastolic blood pressure
EASR	European age standardised rate
ECG	electrocardiogram
eGFR	estimated glomerular filtration rate
ELF	Enhanced Liver Fibrosis panel
ET2DS	Edinburgh Type 2 Diabetes Study
FIB4	Fibrosis-4 Index
GGT	gamma-glutamyl transferase
GP	General Practitioner
GUCI	Goteburg University Cirrhosis Index
HA	hyaluronic acid
HCC	hepatocellular carcinoma
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HR	hazard ratio
ICD	International Classification of Diseases
IQR	inter-quartile range
LDL	low density lipoproteins

LDR	Lothian Diabetes Register
LSM	liver stiffness measure
MRE	magnetic resonance elastography
NAFL	non-alcoholic fatty liver
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD Activity Score
NASH	non-alcoholic steatohepatitis
NFS	NAFLD Fibrosis Score
NHS	National Health Service
NPV	negative predictive value
N-Score	Nippon-Score
OAHA	oral antihyperglycaemic agent
OELF	Original European Liver Fibrosis panel
OR	odds ratio
P3NP	aminoterminal peptide of pro-collagen III
PAF	Probability of Advanced Fibrosis
PIVENS	Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with NASH trial
PPV	positive predictive value
sBP	systolic blood pressure
SIMD	Scottish Index of Multiple Deprivation
SMR	standardised mortality rate
SPEA	serum prolidase enzyme activity
TG	triglycerides
TGFβ	transforming growth factor beta
TIMP-1	tissue metallopeptidase inhibitor 1
TNFα	tumour necrosis factor-α
TUE	transient ultrasound elastography
TZD	thiozolidinedione
ULN	upper limit of normal
USS	ultrasound scan

CHAPTER 1 Background

1.1 Type 2 diabetes mellitus

Diabetes mellitus encompasses a number of metabolic disorders all resulting in hyperglycaemia due to defects in insulin secretion, insulin action, or both. In turn, this chronic hyperglycaemia is associated with long-term damage, dysfunction, and failure of multiple organs. Diabetes can be classified (Appendix A) dependant on the underlying cause e.g. type 1 diabetes – insulin deficiency. This thesis will focus on type 2 diabetes only.

1.1.1 Aetiology

Type 2 diabetes is characterised by impaired pancreatic β-cell function and insulin resistance. Insulin is a hormone released from the β-cells of the pancreas to help control glucose metabolism. Failure to respond to insulin leads to increased levels of circulating glucose. In response the β-cells increase insulin production, however, this ultimately becomes insufficient and a chronic hyperglycaemic state (diabetes) is reached.

Whilst type 2 diabetes has traditionally been seen as driven by insulin resistance, however genome wide association studies (and meta-analyses) have identified a number of loci associated with reduced β-cell function and a number where the effect on beta cell function is not yet known, however for some genes are expressed in the pancreas[1-3].

Exact causes of type 2 diabetes are unclear. One concept is the ectopic fat theory where by obesity leads to ectopic fat deposition that drives organ specific insulin resistance[4].

1.1.2 The ectopic fat theory

The ‘normal’ place for fatty acid storage is in adipocytes as triglyceride. Ectopic fat refers to the accumulation of excess intracellular lipid outside of these typical fat

stores. Lipid oversupply leads first to deposition in subcutaneous adipose tissue, visceral adipose tissue and then into viscera such as the liver and skeletal muscle.

The causes of ectopic fat deposition of three-fold: adipose tissue dysfunction, fat oxidation dysfunction and inflammation. Adipocyte dysfunction occurs when adipocyte tissue mass increases[5], but not adipocyte number. The pattern of adipokine secretion then changes: leptin secretion increases, adiponectin secretion decreases, and chemokine secretion increases to recruit macrophages into the tissue leading to adipose tissue inflammation[6]. These large fat cells are unable to sequester the excess energy as triglyceride which leads to insulin resistance and type 2 diabetes[7,8]. Ideally the increased supply of lipid from dysfunctional adipose tissue is sequested as triglyceride through mitochondrial oxidation of lipid. However, there is evidence of mitochondrial dysfunction and reduced mitochondrial volume in skeletal muscle in type 2 diabetes[9], associated with increasing insulin resistance.

The consequences of this ectopic deposition include insulin resistance, pancreatic β-cell failure and increased very-low density lipoprotein cholesterol production. It has been shown that skeletal muscle fat deposition (intramyocellular lipid) is directly correlated with insulin sensitivity, with increased insulin resistance related to increased ectopic intramyocellular lipid[10]. In the case of people with type 2 diabetes this is due to decreased oxidative capacity. The β-cell is also susceptible to the effects of increased lipid supply. Lipotoxicity leads to β-cell apoptosis and failure of insulin production[11,12]. Hepatic lipid accumulation is associated with increased very-low density lipoprotein production; hepatic insulin resistance[13,14] and non-alcoholic steatohepatitis (NASH) and NASH-cirrhosis. Not all visceral ectopic fat is 'bad'. Cross-sectional data suggest that gluteal adipose tissue is protective and not associated with the same health risks as abdominal fat[15].

1.1.3 Prevalence

The most recent figures from the diabetes Managed Clinical Networks in Scotland[16] report 236,605 patients with type 2 diabetes in Scotland at the end of 2013, 88% of all diabetes cases. In the Lothian region there were 31,833 (crude prevalence 3.8%). There has been a year-on-year increase in the crude prevalence over at least the last

seven years, most likely due to: increased surveillance for and diagnosis of type 2 diabetes; improved data linkage and recording of known cases; and a true increase in the underlying prevalence.

Type 2 diabetes typically affects older people with more than 70% occurring in people aged 60 years or over in Scotland. The current overall incidence of type 2 diabetes in Scotland is 3.4/1000 population/year, rising in those aged 60-69 years to 8.3/1000 population/year and to 7.6/1000 population/year in those aged 70 years and over. Males constitute slightly more of the type 2 diabetes cases than females (55.5%)[16].

1.1.4 Complications

Type 2 diabetes has a number of well acknowledged micro- and macrovascular complications. More recently it has been linked with non-alcoholic fatty liver disease (NAFL) and non-alcoholic fatty liver disease (NAFLD)[17].

1.2 Chronic liver disease

1.2.1 Aetiology

There are a wide range of causes of chronic liver disease (CLD) (Table 1-1).

1.2.2 Prevalence

It is difficult to determine the prevalence of CLD as there is no agreed definition of CLD for collecting data. The recent Lancet Commission – *Addressing liver disease in the UK* – reported that unlike other chronic diseases the standardised mortality rate for liver disease has increased 400% since 1970 and hospital admissions have increased 62% in just 10 years, and that this rise is in contrast to many other European countries where rates are falling[18].

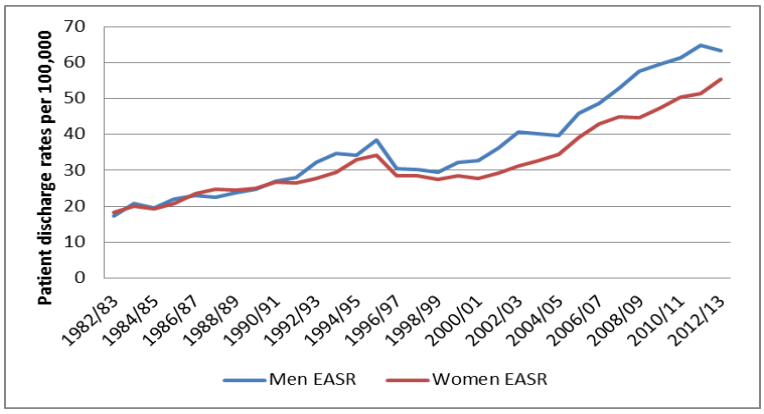
Table 1-1 Causes of chronic liver disease

Long term alcohol consumption
Viral hepatitis (B and C)
Use of certain drugs e.g. amiodarone, methotrexate, glucocorticoids
Chemical exposure e.g. carbon tetrachloride, N,N-Dimethylformamide
Bile duct obstruction
Autoimmune diseases e.g. autoimmune hepatitis, primary biliary cirrhosis
Obstruction of outflow of blood from the liver (for example, Budd-Chiari syndrome)
Heart and blood vessel disturbances e.g. congestive cardiac failure,
Alpha1-antitrypsin deficiency
High blood galactose levels
High blood tyrosine levels at birth
Glycogen storage disease
Cystic fibrosis
Diabetes/obesity/non-alcoholic fatty liver +/- disease
Malnutrition
Wilson's disease (hereditary copper accumulation)
Haemochromatosis (hereditary iron accumulation)

The Scottish Public Health Observatory data collection has defined CLD as International Classification of Diseases (ICD) codes: ICD-9 571.0-571.6, ICD-10 K70 (alcoholic liver disease, ALD), K73 (chronic hepatitis not classified elsewhere), and K74 (fibrosis and cirrhosis of liver)[19]. It does not include chronic viral hepatitis (ICD-10 B18 codes), fatty liver (K76.0), haemochromatosis (E83.1), or complications such as hepatocellular carcinoma (HCC, C22.0).

CLD is mainly treated in the community or through hospital out-patient clinics with much disease being asymptomatic until the later stages. Whilst only the tip of the ice-berg in terms of disease burden, admissions to hospital in Scotland coded through hospital statistics as CLD have been rising in both men and women over the past 20 years. Interestingly associated mortality rose and has since began to fall again (Figure 1-1, Figure 1-2 and Figure 1-3).

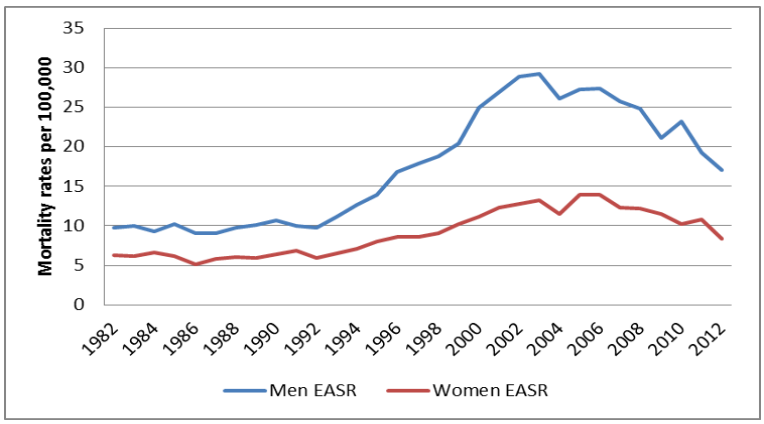
Figure 1-1 Chronic liver disease hospital discharge rates in Scotland 1982-2013



EASR European Age Standardised Rate

Data from Information Services Division, NHS National Services Scotland[19]

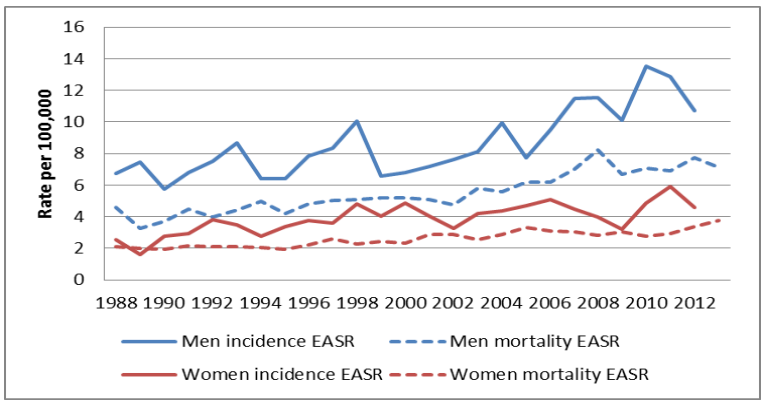
Figure 1-2 Chronic liver disease mortality rates in Scotland 1982-2012



EASR European Age Standardised Rate

Data from Information Services Division, NHS National Services Scotland[19]

Figure 1-3 Cancer of the liver and intrahepatic bile ducts: Incidence and mortality rates in Scotland, 1988-2012



EASR European Age Standardised Rate

Data from Information Services Division, NHS NSS[20]

1.2.3 Pathogenesis of fibrosis and cirrhosis

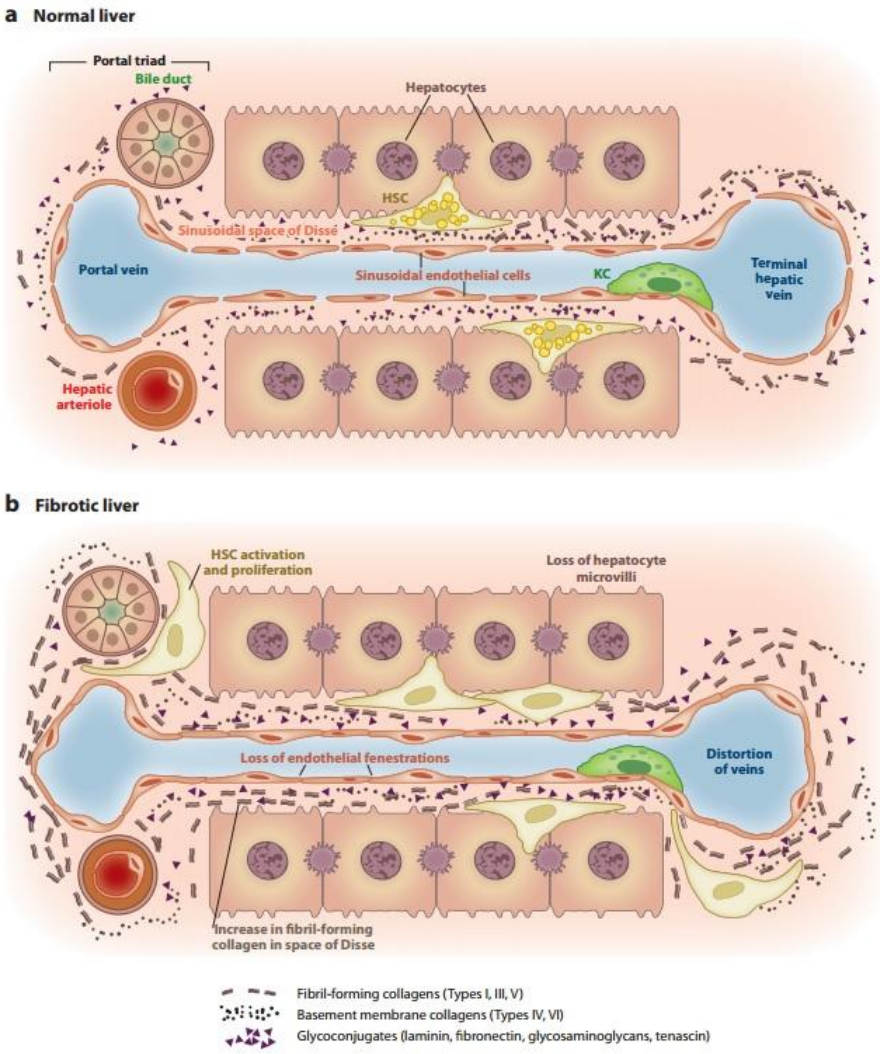
Following any chronic injury to the liver there is a stereotypical cellular response resulting in by the accumulation of excessive amounts of extracellular matrix. If the injury is short-lived then the extracellular matrix can remodel and normal liver architecture is restored, however, if the insult becomes chronic then accumulation of scar extracellular matrix continues with progressive disruption of liver structure and function[21].

The liver parenchyma is made up of: hepatocytes and cholangiocytes (epithelial cells), sinusoidal endothelial cells and other non-parenchymal cells including hepatic stellate cells and Kupffer cells. Figure 1-4a illustrates the structure of normal liver with the endothelial lining separated from the hepatocytes by the subendothelial space of Disse, where the hepatic stellate cells can be found. The space of Disse contains low-density matrix that is both porous enough to allow metabolic exchange between the bloodstream and hepatocytes but dense enough to maintain the different structural areas.

Progressive deposition of extracellular matrix in the space of Disse as a result of chronic liver injury reduces the endothelial porosity ('capillarisation' of the sinusoids) and impairs hepatic function (Figure 1-4b). Different underlying CLD aetiologies result in differing patterns of liver fibrosis (Figure 1-5). For example chronic viral hepatitis typically cause portal-central bridging fibrosis and ALD and NAFLD are characterised by peri-cellular fibrosis, having a characteristic chicken-wire pattern.

As fibrosis progresses to cirrhosis the scar tissue results in distortion of both the liver vasculature and parenchyma. Nodules of regenerating liver tissue can be seen enclosed within fibrotic septae. These events result in significant impairment of liver function with porto-systemic shunting and venous occlusion often occurring and leading to portal hypertension (Figure 1-6).

Figure 1-4 Liver structure



Taken from Hernandez-Gea and Friedman 2011[22]

There is increasing evidence (experimental models and human studies) that liver fibrosis is reversible and even cirrhosis can regress, although heavily cross-linked collagen and certain angio-architectural changes (e.g. vascularised fibrotic septae) may be irreversible[21,23,24].

Figure 1-5 Patterns of liver fibrosis by aetiology

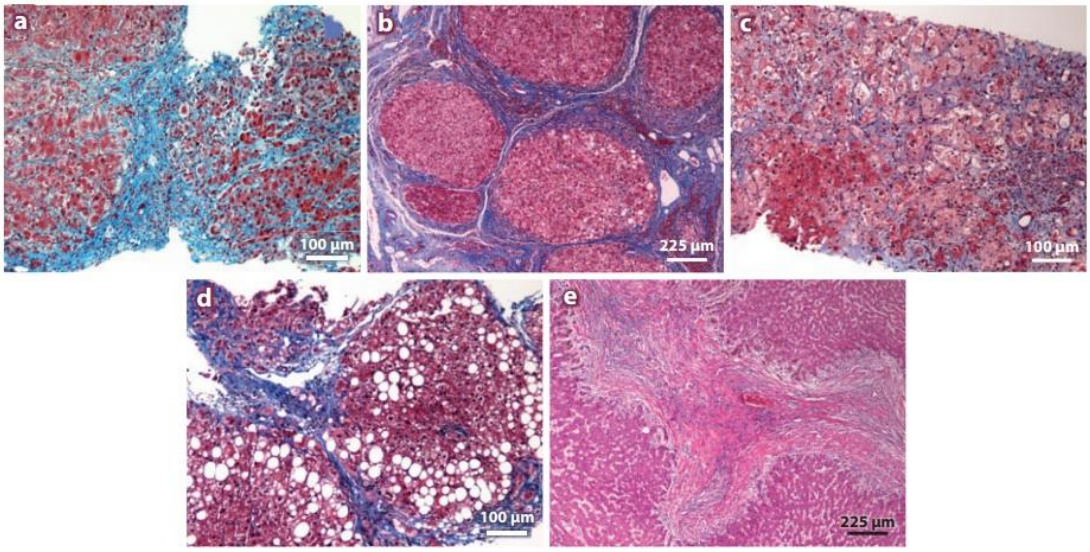
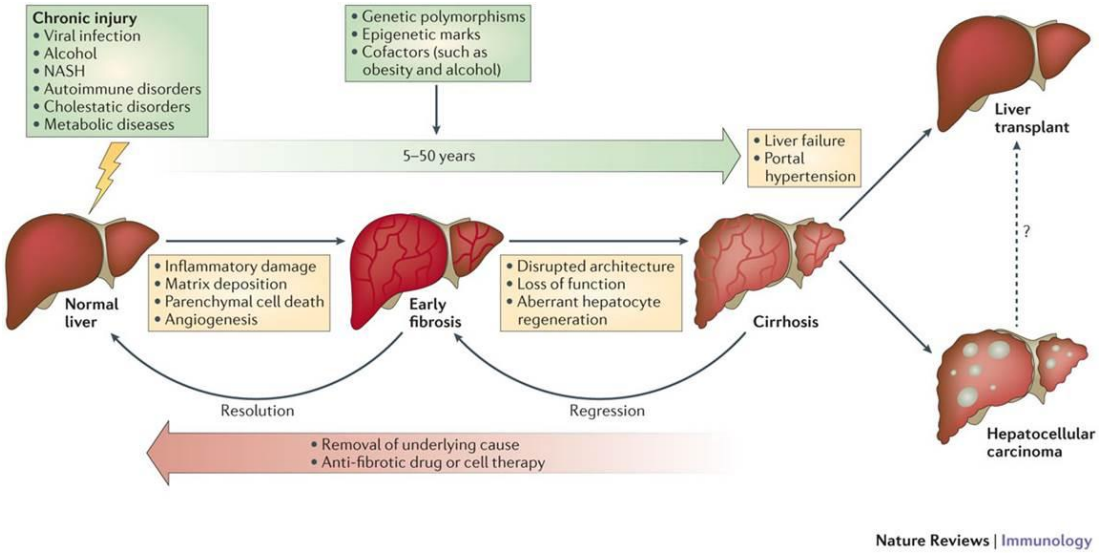


Figure 1

Photomicrographs of fibrosis patterns in different etiologies of liver disease. (*a*) Autoimmune hepatitis. Portal-central vein bridging necrosis. (*b*) Chronic viral hepatitis C. Trichrome staining showing portal-central fibrotic septa and nodule formation. (*c*) Acute alcoholic hepatitis. Deposition of extracellular matrix around hepatocytes (so-called chicken-wire pattern) and ballooning degeneration of hepatocytes. (*d*) Nonalcoholic steatohepatitis. Trichrome staining showing macrovesicular steatosis and pericellular fibrosis. (*e*) Biliary cirrhosis. Portal-portal fibrotic septa and proliferation of bile ductules. Images reproduced courtesy of Dr. M. Isabel Fiel, Mount Sinai School of Medicine.

Taken from Hernandez-Gea and Friedman 2011[22]

Figure 1-6 The natural history of chronic liver disease



Taken from Pellicoro et al 2014[21]

1.2.4 Complications of chronic liver disease – hepatic decompensation

Compensated cirrhosis means that the scarred and damaged liver is still able to undertake most of its functions. Patients may be asymptomatic. Ultimately the liver loses its ability to maintain normal function when the damage becomes too extensive or when further challenged (e.g during a period of infection, dehydration, constipation) – termed hepatic decompensation.

Portal hypertension, hypersplenism and gastro-oesophageal varices

Portal hypertension is an increase in the blood pressure within the portal venous system. Portal hypertension can be classified according to the anatomic site of increased resistance to portal blood flow: pre-hepatic, intrahepatic and post-hepatic. In cirrhosis, architectural (structural) changes in the chronically injured liver are responsible for 60-70% of the increase in intra-hepatic vascular resistance that initiates portal hypertension[25]. Contraction of peri-sinusoidal myofibroblasts (mostly derived from activated hepatic stellate cells) contributes to 30-40% of the increase in intra-hepatic vascular resistance, and is mainly due to a deficiency in hepatic nitric oxide levels. In addition, splanchnic arteriolar vasodilation and a hyper-dynamic circulation increase portal blood flow and aggravate portal hypertension[26]. Clinically significant portal hypertension is defined as a hepatic venous pressure gradient $\geq$10 mmHg. The consequences of portal hypertension include hypersplenism, development of gastro-oesophageal varices and ascites. Variceal bleeding is the last step in a chain of events initiated by an increase in portal pressure, followed by the development and progressive dilation of varices until these finally rupture and bleed. It has been estimated that varices are present in about 30–40% of compensated cirrhotic patients at the time of diagnosis, and in 60% of decompensated cirrhotic patients[27-29].

Thrombocytopenia and coagulopathies

Splenic enlargement occurs as a result of portal hypertension. Thrombocytopaenia (platelet count $<150x10^9$/L) may be present in as many as in 76% of patients with cirrhosis[30] – occurring partly due to the increased pooling of platelets in the enlarged spleen, and also due to suppressed bone marrow production. Furthermore, immunosuppressive medication and anti-viral therapy can also induce

thrombocytopaenia. In addition, patients with cirrhosis develop coagulopathy due to loss of synthetic function. This is evidenced as a reduction in the production of: clotting factors (I (fibrinogen), II (prothrombin), V, VII, IX, X, XI, protein C, and anti-thrombin) and increased bleeding risk[31].

Hepatorenal syndrome

The initial change in renal function is the reduced capacity to excrete sodium, resulting in the accumulation of sodium and water in the abdominal cavity (ascites). The reabsorption of sodium is driven by i) the up-regulation of the renin-angiotensin-aldosterone system, and ii) over-activity of the sympathetic nervous system. In addition, free water excretion by the kidney is reduced. The final change in renal function is renal vasoconstriction leading to reduced renal blood flow and a fall in glomerular filtration rate[32]. The diagnostic criteria for hepatorenal syndrome include a number of major and minor criteria. Major criteria: portal hypertension; renal failure; the absence of shock; infection; recent treatment with nephrotoxic medications and fluid losses; the absence of sustained improvement in renal function despite treatment with 1.5 litres of intravenous normal saline; the absence of proteinuria; and, the absence of renal disease or obstruction of renal outflow as seen on ultrasound. Minor criteria: a low urine volume (less than 500 mL per day), low urinary sodium concentration, urine osmolality > blood osmolality, the absence of red blood cells in the urine, and a serum sodium concentration of less than 130 mmol/L[33].

Hepatopulmonary syndrome

Hepatopulmonary syndrome involves the formation of arteriovenous dilations in the intrapulmonary vasculature, leading to over-perfusion relative to ventilation, and hypoxaemia. The severity of hepatopulmonary syndrome is based on the oxygenation deficit rated from mild, with an alveolar–arterial oxygen gradient ≥15 mmHg and a partial pressure of oxygen ≥80 mmHg, through moderate and severe, to very severe with a partial pressure of oxygen <50 mmHg[34,35].

Cirrhotic cardiomyopathy

Cirrhosis also causes changes in the systemic circulation. A hyper-dynamic circulation results from reduced systemic vascular resistance and arterial vasodilation. In addition, pooling of the blood in the splanchnic circulation results in effective central hypovolaemia, which is followed by baroreceptor-induced activation of renin-angiotensin-aldosterone-system and the sympathetic nervous system. In response there is an attempt at increasing and maintaining cardiac output[36-38]. There is a further suggestion that cirrhosis itself may have an influence on the heart – although this is unclear. The result of these events is a cardiac condition characterized by a reduced contractility, diastolic dysfunction and electromechanical abnormalities, in the absence of any other cardiac disease[39,40]. These cardiac changes have been reported to occur in up to 40–50% of patients with cirrhosis[41,42].

Hepatocellular carcinoma

It is thought that approximately a third of patients with cirrhosis will develop HCC at some point and that 90% of patients with HCC have cirrhosis[43]. Screening for HCC is recommended for patients with: cirrhosis and Child-Pugh stage A or B; cirrhosis and Child-Pugh stage C awaiting liver transplant; non-cirrhotic hepatitis B virus carriers with active hepatitis or family history of HCC; and non-cirrhotic chronic hepatitis C virus patients with Metavir stage F3+ liver fibrosis[44].

Hepatic encephalopathy

Hepatic encephalopathy encompasses a range of reversible neuropsychiatric abnormalities as a result of the livers inability to remove toxins (e.g. nitrogenous substances derived from the gut) from the portal blood[45-47]. In the brain these substances alter neurotransmission affecting conscious level and behaviour. hepatic encepalopathy is graded as: Grade 1 - trivial lack of awareness, euphoria or anxiety, shortened attention span, impaired performance of addition; Grade 2 - lethargy or apathy, minimal disorientation for time or place, subtle personality change, inappropriate behaviour, impaired performance of subtraction; Grade 3 - somnolence to semi-stupor, but responsive to verbal stimuli, confusion, gross disorientation; and Grade 4 - coma (unresponsive to verbal or noxious stimuli)[48]. Consensus

terminology[49] defines hepatic encepalopathy as: hepatic encepalopathy associated with acute liver failure; hepatic encepalopathy associated with porto-systemic bypass without intrinsic hepatocellular disease; and hepatic encepalopathy associated with cirrhosis and/or portal hypertension. This third group can be further subdivided into episodic, persistent and minimal. Treatment of hepatic encepalopathy is centred on supportive care, identification and removal of precipitants (e.g. infection, dehydration, renal failure, constipation, gastro-oesophageal haemorrhage), reduction of the nitrogenous load from the gut, and assessment of the need for long term risk reduction therapy (e.g. non-absorbable antibiotics)[50].

Malnutrition

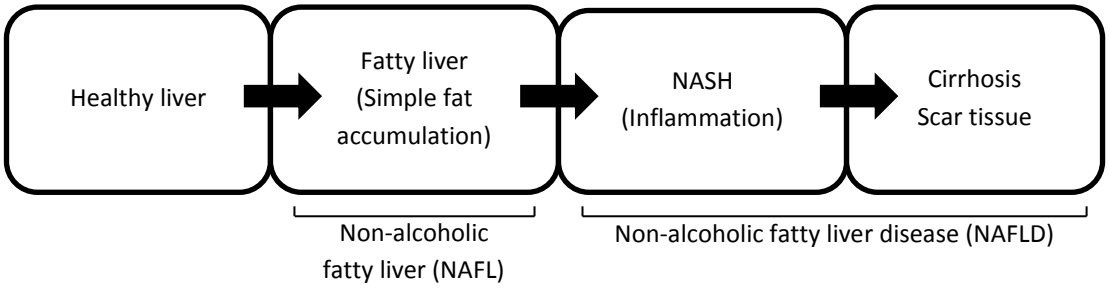
The prevalence of malnutrition in cirrhosis is as high as 65%–90%[51,52]. A variety of processes contribute to malnutrition in cirrhotic patients: poor dietary intake, malabsorption, increased intestinal protein loses, reduced protein synthetic function, disturbances in substrate use, and a hypermetabolic state[53].

1.3 Non-alcoholic fatty liver and non-alcoholic fatty liver disease

1.3.1 Terminology

The terminology used to describe non-alcoholic fatty liver (NAFL) and non-alcoholic fatty liver disease (NAFLD) can be confusing. Until relatively recently the term NAFLD was used to include all stages of the disease process and this frequently remains the case. In 2012, The American Association for the Study of Liver Diseases published an updated practice guideline separating the spectrum of changes within the liver into two parts: 1) benign non-alcoholic fatty liver with hepatosteatosis only – NAFL, and 2) bringing necro-inflammatory non-alcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis together to form the 'disease' element of the spectrum – NAFLD (see Figure 1-7). This was in order to highlight the more 'clinically significant disease'.

Figure 1-7 The natural history of non-alcoholic fatty liver disease



As such, throughout this thesis the following terms will be used:

- NAFL, non-alcoholic fatty liver – the presence of hepatic steatosis only
- NAFLD, non-alcoholic fatty liver disease – the presence of NASH, hepatic fibrosis or cirrhosis
- NAFL/D, the full spectrum of liver disorder – NAFL and NAFLD combined.

1.3.2 Prevalence

The unadjusted prevalence of NAFL/D in the general population is estimated to be between 9-46%[54-58] with the wide variation in reported results being due to the population studied and diagnostic criteria used. This wide range is due to NAFL/D being diagnosed using elevated liver enzymes, USS or liver biopsy. The most plausible estimates come from USS and other imaging studies as whilst not having the diagnostic accuracy of liver biopsy, USS is non-invasive and can be used widely in general populations unlike liver biopsy which is typically restricted to patients with a high suspicion of disease. It is generally accepted that the prevalence is rising (although there are no definitive confirmation of this) and that the driving factors behind this are diabetes and obesity.

One of the most plausible estimates for the prevalence of NAFL/D include those provided by the Dallas Heart Study (a multi-ethnic population-based sample)[59] which used high resolution magnetic resonance spectroscopy to measure hepatic TG. With NAFL/D defined as hepatic steatosis (hepatic TG content >5.5%) in the presence of no alcohol consumption the prevalence was29.3% (670/2287)[60].

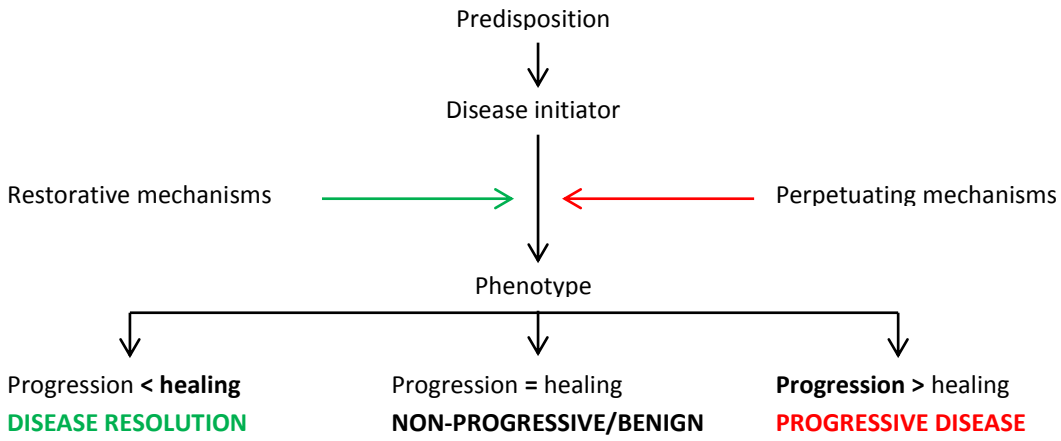
What is more difficult to determine is the prevalence of NASH and related fibrosis. Studies are limited by the need for liver biopsy (the current controversial gold standard) with understandably few undertaken in the general population who are typically asymptomatic.

The overall prevalence of NAFL/D is quoted as up to 70%[61], however the studies providing these estimates are frequently restricted to selected populations. For example, a recent study of a largely military population in the USA found a NAFL/D prevalence of 46% and a NASH prevalence of 12%[55].

1.3.3 Pathogenesis

The initial pathological hypotheses, the 'double hit' hypothesis[62] proposed by Day et al in 1998 was highly influential in the understanding the pathogenesis of NAFL/D. It has since been superseded by the 'multiple parallel hits' hypothesis[63] however, they have a number of elements in common, and both surmise that the development of NAFL/D is a multifactorial disease, both genetic and environmental in origin, with both perpetuating and restorative events repeatedly occurring over time (Figure 1-8).

Figure 1-8 Development, progression and regression of non-alcoholic fatty liver /disease



Non-alcoholic fatty liver

The first stage of the development of NAFL/D is the accumulation of adipose tissue within the liver parenchyma (steatosis), known as NAFL. Figure 1-9 shows the pathophysiological process of NAFL/D.

There are three key drivers of this process: i) increased free fatty acid influx into the liver; ii) de novo lipogenesis within the liver; and iii) reduced export of fat from the liver.

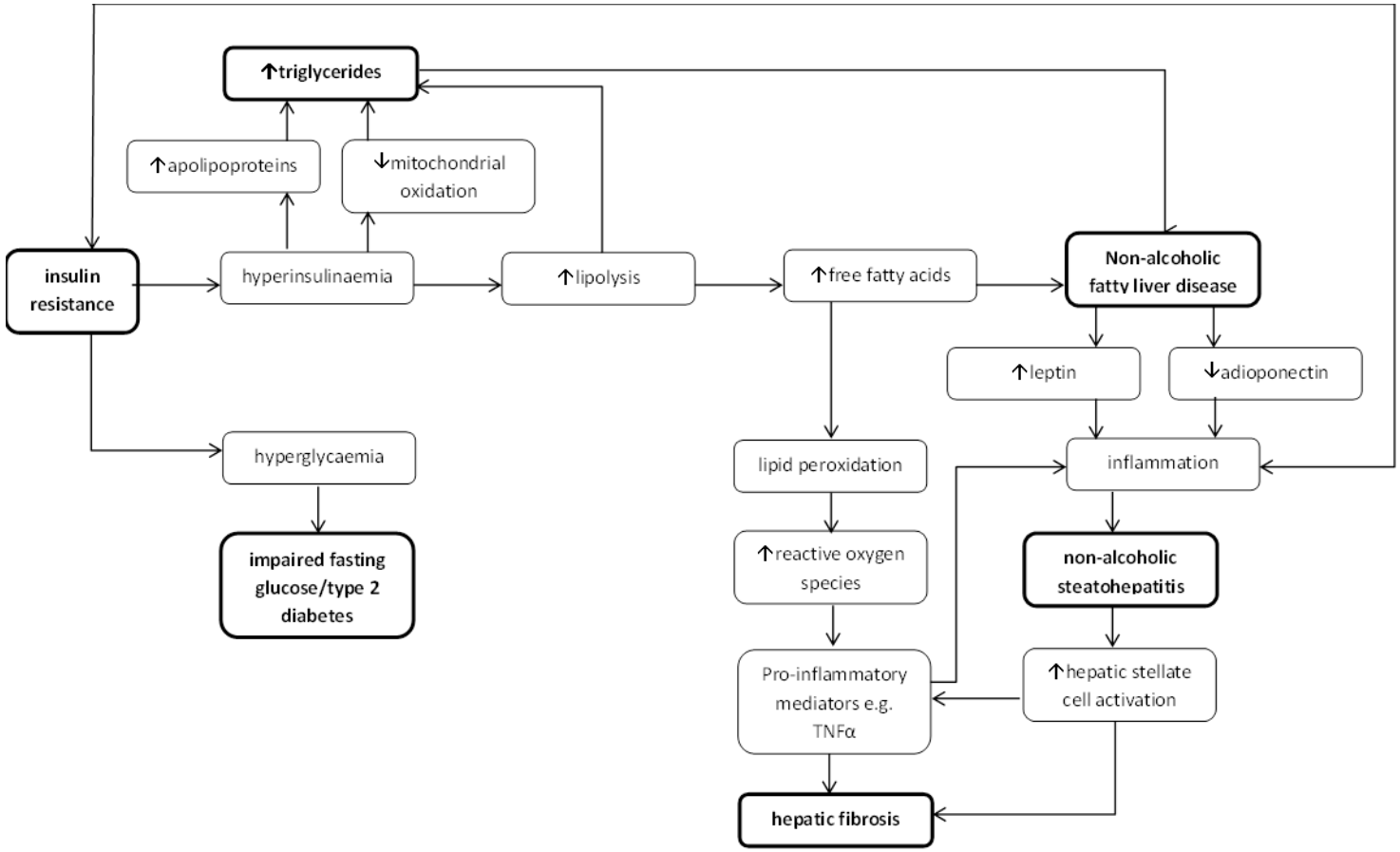
The core underlying premise is that a series of events occurs in response to insulin resistance and resultant hyperinsulinaemia. Ectopic hepatic steatosis is thought to occur due to an increase in hepatic free fatty acids driven by insulin resistance through: the hydrolysis of triglycerides (TG) in peripheral adipocytes, hydrolysis of dietary TG, and also endogenous free fatty acid synthesis[64,65].

Insulin triggers hepatic lipogenesis through sterol regulatory element-binding protein-1c, carbohydrate response element-binding protein, and peroxisome proliferators-activated receptor-gamma[64,66].

In addition, hyperinsulinaemia results in higher circulating levels of TG in the form of very low density lipoproteins due to increased delivery of free fatty acids to the liver though impaired inhibition of hormone-sensitive lipase, and reduced hepatic TG secretion through impaired mitochondrial β-oxidation[67,68].

NAFL is thought to be a largely benign condition that is in itself it doesn't appear to confer any significant hepatic function deficiencies or complications.

Figure 1-9 Pathophysiology of non-alcoholic fatty liver /disease



Non-alcoholic steatohepatitis

The progression of NAFL to inflammation (NASH) and fibrosis/cirrhosis is believed to be a response to i) oxidative stress within the liver and ii) cytokine production.

Usually there is a balance between antioxidants and reactive oxygen species. In normal circumstances, some degree of lipid oxidation occurs to prevent excess lipid being deposited in the liver. In progressive NAFLD, excessive lipid oxidation occurs causing oxidative stress and reduced levels of antioxidants.

Mitochondria play a key role in the inflammatory process. There are a number of mitochondrial abnormalities present in NASH, namely mega-mitochondria, loss of cristae and the presence of paracrystalline inclusions. These structural abnormalities result in abnormal electron transport chain activity and ultimately the development of reactive oxygen species, which perpetuates a vicious cycle of events. The ineffective electron transport chain activity also leads to increased expression of tumour necrosis factor-α (TNF-α) which also further stimulates lipid peroxidation[63,66].

Lipid peroxidation produces aldehydes, 4-hydroxynonenal and malondialdehyde, which activate hepatic stellate cells. These collagen producing cells lead to the formation of Mallory bodies and neutrophil chemotaxis. Malondialdehyde may also stimulate nuclear factor kappa-light-chain-enhancer of activated B cells which controls expression of further proinflammatory mediators including TNF-α, interleukin-8, intercellular adhesion molecule 1 and E-selectin[63,69].

Liver fibrosis and cirrhosis

The development of fibrosis is a response to chronic liver damage. Quiescent hepatic stellate cells become ‘activated’ in response to persistent injury and undergo a transition to matrix-producing myofibroblast-like cells. They also influence inflammatory cell activity and cytokine production that perpetuate the fibrotic response. The severity of underlying insulin resistance is independently associated with the degree of fibrosis.

1.3.4 Diagnosis

NAFL/D is largely a diagnosis of exclusion. The formal definition provided by the American Association for the Study of Liver Diseases is:

"[T]here is evidence of hepatic steatosis, either by imaging or by histology and there are no causes for secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication or hereditary disorders[61]."

Table 1-2 outlines typical causes of hepatosteatosis that need to be excluded for clause (b) of the definition above, however there is no definitive agreed criteria for these, such that different studies apply for example differing alcohol cut-offs or include different drugs on their list of medications causing hepatic fat accumulation.

Table 1-2 Causes of hepatic steatosis

Alcohol	Typically >20g/day (2.5 units) females or >30g/day (3.8 units) males [70]
Insulin resistance	Lipoatrophy Mauriac syndrome
Disorders of lipid metabolism	Abetalipoproteinaemia, hypobetalipoproteinemia, Anderson's disease, Weber-Christian syndrome, acute fatty liver of pregnancy, Wolman's disease, Wilson's disease, Reyes syndrome, Dorfman Chanarin syndrome
Nutritional	Total parenteral nutrition, severe weight loss (jejunoileal bypass, gastric bypass, severe starvation), refeeding syndrome, protein calorie malnutrition, coeliac disease, inflammatory bowel disease
Drugs	Amiodarone, diltiazem, nifedipine, verapamil, synthetic oestrogens, corticosteroids, highly active antiretroviral therapy, tetracycline, methotrexate, sodium valproate, aspirin
Hepatotoxins	Phosphorus, petrochemicals, toxic mushrooms (Amanita phalloides, Lepiota), organic solvents, Bacillus cereus toxin
Infections	Human immunodeficiency virus, hepatitis C virus, small bowel diverticulosis with bacterial overgrowth, gram-negative sepsis

Diagnosis of steatosis

There are a number of methodologies allowing the diagnosis of hepatic steatosis. The validity of each is considered to vary.

The simplest and most commonly used method is abdominal ultrasound scan (USS). Detection of hepatic fat is based on well-established characteristics including a hyperechogenic 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 [71-74]. However there is no definitive consensus on diagnostic criteria[75].

USS is frequently cited as having poor diagnostic accuracy in the identification of hepatic steatosis. A 2011 meta-analysis determined the sensitivity of ultrasound in diagnosis of any degree of hepatic steatosis was 73.3% and corresponding specificity was 84.4%[76]. However, the diagnostic accuracy of included studies varied with sensitivity as low as 53% and specificity as low as 77%. Some of this variation may be explained by the varied populations under study and by variation in diagnostic criteria used.

The non-invasive gold-standard for the detection of hepatic steatosis is magnetic resonance spectroscopy. Different "normal" values for hepatic fat fraction on magnetic resonance imaging have been reported but typically are attributed to ≤5% fat[60,77,78]. A recent meta-analysis found magnetic resonance spectroscopy to be superior to both computed tomography and USS for the diagnosis of hepatic steatosis with sensitivity and specificity of 89% and 92% respectively for a fat fraction of up to 5% steatosis, and of 73% and 96% respectively for a fat fraction above 25%[77].

Diagnosis of non-alcoholic steatohepatitis and liver fibrosis

Currently the only universally accepted method (and gold standard) for the diagnosis of the inflammatory and fibrotic stages of NAFLD is liver biopsy to allow histological assessment. Numerous non-invasive methods have been introduced over recent years. Suggested markers of NASH include cytokeratin-18 (CK18), adipocyte fatty acid binding protein, fibroblast growth factor 21 and NASHTest[79-81]. CK18 has

been most extensively investigated. Across 6 studies involving a total of 561 participants (range 44-139) the diagnostic accuracy of CK18 (vs liver biopsy) as determined by the area under received operating characteristic (AUROC) to be 0.71-0.93. The associated sensitivities and specificities for the optimal cut off to determine NASH in each study were 62-86% and 81-100% respectively. It should be noted that all of the studies used secondary/tertiary care based populations with a high prevalence of NASH[82-87].

Non-invasive markers of liver fibrosis are reviewed in detail in Chapter 2.

1.3.5 Risk factors

Age

NAFL/D is found in all age groups including children, however there is an increasing prevalence with increasing age[60]. For example, a general population post mortem study found the prevalence of fatty liver in the was 1% in people below 20 years, 18% between 20 and 40 years, and 39% among 60 and older[88].

Ethnicity

Differing ethnicities bring differing risk profiles for NAFL/D.

Browning et al found that African Americans have significantly less magnetic resonance spectrospcopy measured hepatic TG (median 3.2%) and hepatic steatosis (24%) than non-Hispanic whites (3.6% and 33%) or Hispanics (4.6% and 45%) even after adjusting for obesity and diabetes[60].

Asian populations have a higher prevalence of type 2 diabetes and insulin resistance than Caucasian populations which is directly reflected in their higher NAFLD prevalence[89,90].

Genetics

A number of different studies have suggested that there is a heritable component to NAFL/D: family aggregation studies[91-93], twin studies[94] and the above mentioned ethnic differences[60,95,96].

A number of genetic associations have been observed. The first genome wide association study by Romeo et al in 2008[97] identified a single highly significant association with increased hepatic TG levels for the PNPLA3 gene. A gene dose effect for the index SNP (rs738409) was observed. Following this PNPLA3 remains the most validated gene associated with all aspects of the NAFL/D spectrum[98-103].

Sex

Reports are very mixed on the distribution of NAFL/D between men and women. Initial reports suggested NAFL/D was more common in females, with a shift now to a more even distribution or possibility of an increased prevalence in males. NAFL/D is more common is post-menopausal women than pre-menopausal[104], and hormone replacement therapy appears protective[105]. Sex-related fat distribution differences mean that females tend to have less visceral fat and this may explain a reduced prevalence compared to males[106].

Obesity

Obesity is a recognised risk factor for NAFL/D, even after adjustment for insulin resistance. As a response to excess calorific intake the adipocytes hypertrophy followed by hyperplasia (fat cell replication). As described in section 1.3.3, in an attempt to avoid excess hepatic TG accumulation the adipocytes activate a number of inflammatory pathways potentially leading to NASH.

Type 2 diabetes

The relationship between type 2 diabetes and NAFL/D is discussed in section 1.5.

1.3.6 Disease progression

Given that hepatic steatosis is considered to be a benign condition for the majority of patients, of more interest is (a) the rate of progression to NASH and to liver fibrosis and cirrhosis, and (b) any evidence of reversal from the later stages back to simple steatosis. The recent Lancet Commission – *Addressing liver disease in the UK* – suggested that a third of obese UK individuals have NAFL/D, and in almost one in

ten, over the course of 20-50 years, it will result in cirrhosis and its related complications[18].

A recent meta-analysis[107] of ten studies (221 subjects) of NAFL/D progression found 37% progressed to a higher fibrosis category (Metavir F1+ disease 0.41 stages/year, overall 0.03 Metavir stages/year) and 21% improved their fibrosis classification during a median follow-op period of 3.7 years (range 1.0-21.3 years). Further analysis found that age (younger) and the presence of inflammation on the diagnostic biopsy were independent predictors of this progression. Expected risk factors (e.g. diabetes and obesity) were not statistically significant. Table 1-3 summarises the data on histological changes in NAFL/D from each of the studies contributing to the fore-mentioned meta-analysis and the additional studies identified. Caution is required in the interpretation and comparison of these studies for a number of reasons: (a) there was a different baseline prevalence of NASH stages and fibrosis scores in each study, thus the %change does not reflect the baseline number able to change (ie a study with predominantly F0 and F1 subjects at baseline will by definition have a low proportion regressing); (b) some of the studies were appended to intervention studies; and (c) all of the studies were based on biopsy data therefore have been performed in selected populations.

Of note, most of the studies were small (max n=103) with short follow-up periods (50% <5 years). Six studies either reported overall fibrosis change rates or provided sufficient data to allow their calculation. Only one study[108] found the average rate of change to be in the direction of regression. The others had progression rates between 0.03 and 0.11 Metavir fibrosis stages/year. This would translate that a subject with no fibrosis (F0) would take between 36 and 133 years to develop cirrhosis (F4).

Table 1-3 Progression and regression of pathological changes in non-alcoholic fatty liver /disease

Study	*Year*[a]	*Country*	*N*	*Age, years*[b]	*Male, %*	*Follow-up period, years*[b]	*NASH progression, %*	*NASH regression, %*	*Fibrosis progression, %*	*Fibrosis regression, %*	*Overall rate of fibrosis change, stages/year*
Adams[109]	1980/2003	USA	103	45.0 (11.5)	37	3.2 (3.0)	-	-	36.9	29.1	0.09
Argo[108]	1997/	USA	5	-	-	4.4 (1.9)	-	-	60.0	20.0	-0.07
Ekstedt[113]	1988/2003	Sweden	70	51.0 (12.9)	67	13.8 (1.2)	-	-	41.4	15.7	-
Evans[114]	1985/1999	UK	7	57.5 (9.1)	43	8.2 (2.6)	0	42.9	57.1	0	0.09
Fassio[115]	1986/2002	Argentina	22	44.7 (12.7)	41	4.3 (3.0-14.3)	-	-	31.8	18.2	0.06
Hamaguchi[112]	1997/2008	Japan	39	47 (20-79)	56	2.4 (1.0-8.5)	23.1	17.9	28.2	30.7	-
Harrison[116]	1985/2001	USA	22	50.6	59	5.7	18.2	45.5	31.8	18.2	-
Hui[117]	1996/2004	Hong Kong	17	41.8 (2.6)	65	6.1 (3.8-8.0)	11.8	23.5	52.9	0	0.11
Chan[118]	2014	Kuala Lumpar	35	47.5 (10.9)	40	6.4 (0.8)	37.1	37.1	51.4	0	
Pais[111]	1998/2009	France	70	52 (10.5)	-	3.7 (2.1)	41.4	31.4	28.6	28.6	-
Ratziu[119]	1988/1999	France	14	-	-	5.2 (3.9)	-	-	14.3	-	-
Wong[110]	2006/2009	Hong Kong	52	44 (9)	65	3	38.5	19.2	26.9	25.0	0.03

[a]year recruitment commenced/year follow-up finished or year of publication; [b]mean (sd) or median (range)

NASH non-alcoholic steatohepatitis.

In addition, whilst many of the studies found univariate associations between potential risk factor for and fibrosis change, few reported independent associations after adjustment in multivariable analysis. Those that did reported progression of fibrosis associated with increasing baseline body mass index (BMI, p=0.008)[109], presence of baseline obesity (p=0.005)[109], increase in waist circumference between biopsies (p=0.002)[110], increased baseline low-density lipoprotein (LDL) cholesterol (p=0.019)[110] and increased quantitative steatosis measurement on baseline biopsy (p=0.001)[111]. Regression of liver fibrosis was independently associated with >1% improvement in HbA1c between biopsies (p=0.01)[112] and insulin treatment at baseline (p=0.03)[112].

1.3.7 Treatment

At present there is no pharmacological therapy licenced for the treatment of NAFL/D. Research into treatments is focusing on two main effects (i) early disease: regression/halting the progression of steatosis and/or NASH, and (ii) later disease: anti-fibrotic therapies. Clinical trials are limited by the lack of a non-invasive marker that aligns with the histological grade of disease to allow monitoring of disease progression/regression. There is also no universally agreed endpoint/outcome for trials. Currently, histological changes seen on liver biopsy are the suggested end-point for trials of 12-24 month duration, including reduction in NAFLD Activity Score combined with either improvement or lack of progression in fibrosis score (see section 1.4.2).

Insulin resistance is probably the main driver in NAFL/D, however, targeting the steatosis element of NAFL/D is unlikely to be sufficient to treat NASH and fibrosis. In the early stages of NAFL treatment options include lifestyle modification and weight loss, alongside lipid lowering agents. Newer antifibrotic targets include: obeticholic acid (a semi-synthetic derivative of the primary human bile acid chenodeoxycholic acid involved in the regulation of glucose and lipid metabolism); dual peroxisome proliferator-activated receptor α/δ Agonist (regulation of metabolic homeostasis, inflammation, cellular growth and differentiation); anti-LOXL2 monoclonal antibody (an enzyme that promotes cross-linking of fibrillar collagen I);

modulating the gut-liver axis; adiponectin agonists (anti-inflammatory cytokines); glucagon-like peptide 1 analogues (reduces pancreatic insulin secretion following feeding); galectin inhibitors; and C-C chemokine receptor 2 and 5 antagonists. It may be that combined approaches, targeting both inflammatory and fibrogenic pathways, will be need to treat NASH and fibrosis.

Thoma[120] recently undertook a systematic review of lifestyle modification on intrahepatic TG concentration, liver enzymes, and/or insulin sensitivity in adults (⩾19years) with NAFL or NASH. They concluded that lifestyle modifications are effective in reducing intrahepatic TG and circulating liver enzymes, and improving measures of glucose control and/or insulin sensitivity in patients with NAFL/D. They noted that these improvements were driven by weight reduction with weight reductions of 4−14% resulting in statistically significant relative reductions in intrahepatic triacyglycerol of 35−81% but that physical activity could result in (not statistically significant) reductions in intrahepatic TG without a reduction in weight.

Musso[121] performed a meta-analysis examining pharmacological agents for the treatment of NAFL/D. Overall they concluded that most of the 49 included trials were small (median 50 subjects, range 16-247) with a duration of less than one year. Weight loss, thiazolidinediones and antioxidants constituted the majority of trials (10, 8 and 12 respectively). Weight loss had no significant reported side effects and a dose-dependent response was seen with improvement in NASH. However, in excess of 50% of subjects failed to achieve their target weight loss. Thiazolidinediones improved steatosis and inflammation but were not well tolerated (up to 18% drop-outs) due to associated weight gain of 2-5kg in 66-75% of subjects and peripheral oedema in 4-10%. Randomised controlled trials of antioxidant therapies have reported mixed results for improvement in steatopsis, histological NASH and transaminases, and no effect on fibrosis. Improvement in NASH histology in at least one randomised controlled trial was seen with pentoxifylline, telmisartan and L-carnitine. Finally, polyunsaturated fatty acids improved transaminases and steatosis in NAFL/D.

Diabetes therapy and non-alcoholic fatty liver

Several common diabetes treatments have been considered in the treatment of NAFL, due to the shared aetiology (ectopic fat).

Metformin improves hepatic insulin action without affecting hepatic or muscle lipid.

Thiazolidinediones (TZDs) have been shown to modulate ectopic fat (despite often increasing total body fat[122]) through the formation of new adipocytes, storage of lipids in adipose tissue[123,124], increased adiponectin secretion[125], decreased ectopic liver fat[125] and improved insulin sensitivity[125].

Whilst not a diabetes treatment, the majority of patients with type 2 diabetes will be receiving a statin (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors) as advised in national guidelines[126]. Statins act on hepatocytes to inhibit HMG-CoA reductase, reducing the production of cholesterol precursors. The most common hepatic adverse event involves asymptomatic increases in liver transaminases. This dose-dependent reaction usually occurs within the first year of therapy, but may present at any time[127,128]. To date, there is no convincing evidence that lipid-lowering agents, including statins, are beneficial for patients with NAFL.

1.3.8 Extrahepatic complications

Type 2 diabetes

The relationship between type 2 diabetes and NAFL/D is discussed in section 1.5.

Cardiovascular disease

The relationship between cardiovascular disease (CVD) and NAFL/D is discussed in section 1.6.

Renal disease

Several studies have suggested NAFL/D as an independent risk factor for chronic kidney disease (CKD) with studies finding a prevalence of 21-54% compared to 3.7-

24.2% in non-NAFLD patients[129-132]. Even after adjusting for traditional risk factors for CKD (including age, sex, hypertension, diabetes, BMI, dyslipidaemia) NAFL/D on imaging has been found to confer an increased risk with OR 1.87-6.14[129-132]. Although this has not been a universal finding[133]. The risk of developing CKD following a diagnosis of NAFLD is also elevated, HR 1.49-4.38 (after adjustment)[134-137], however, these studies use differing diagnoses of NAFL/D (USS or elevated gammaglutamyl transferase, GGT) and had variable follow-up time.

Colorectal cancer

There is increasing interest in the relationship between NAFL/D and colorectal cancer. Three retrospective studies have found an association between NAFL/D and colorectal adenomas after adjustment (NAFL/D OR 1.45-1.47[138,139], NASH OR 4.89[140]) with one study finding no association[141].

Endocrinopathies

NAFL/D has been associated with growth hormone deficiency, hypogonadism, hypopituitarism, polycystic ovarian syndrome, hypercortisolaemia and hypothyroidism[142] although the evidence is currently limited.

Obstructive sleep apnoea syndrome

There is an increased prevalence of NAFL/D in people with obstructive sleep apnoea independent of age, sex and BMI. In a recent meta-analysis[143] of 18 studies (2183 subjects) Musso et al found pooled OR in obstructive sleep apnoea syndrome for the presence of NAFL/D, as defined by histology, radiology, or transaminase elevation, 2.53 (95%CI 1.93-3.31). Pooled ORs in obstructive sleep apnoea syndrome with severity of NAFL/D liver histology were similar at 2.37 (95%CI 1.59-3.51), 2.16 (95%CI 1.45-3.20) and 2.30 (95%CI 1.21-4.38), for NASH, fibrosis-any stage, or advanced fibrosis respectively.

Osteoporosis

Three studies in adults have found a relationship between the presence of NAFL/D and osteoporosis (bone mineral density measure or fracture assumed to be osteoporotic) after adjustment for known risk factors (including age, sex, BMI,

smoking, alcohol, metabolic syndrome)[144-146]. This risk is appears to be present in both males and females (prevalence of osteoporotic fracture 2.3% in men and 2.9% in women)[145], and also in both adults and children[146].

1.4 Liver biopsy

1.4.1 Procedure

Liver biopsy involves the physical sampling of a small portion of the liver for histological examination. The most common method is percutaneous biopsy where a biopsy needle is advanced through the skin to obtain the sample within a few seconds. The use of USS guidance to facilitate the procedure is now recommended due to its reduced complication rates [147] compared to a 'blind' procedure and resultant reduced costs [148,149]. An alternative approach (when percutaneous is not possible) is via the transjugular route and occasionally laparoscopically.

Complications

Liver biopsy is associated with a small but significant morbidity and mortality with a suggested directly attributable mortality rate of between 1 in 1000 [150] and 1 in 10000 [151] for the percutaneous procedures. The most frequently cited UK figure for the rate of major complications (e.g. haemorrhage, visceral puncture) is 2% based on a study in 1995 [150].

Reliability of liver biopsy in non-alcoholic fatty liver disease

There are justified concerns about the reliability of liver biopsy in NAFL/D. It samples only 1/50,000th of the liver, which is particularly problematic in NAFL/D and NASH because they are often patchy disorders throughout the liver. A recent study by Ratzui looking at reproducibility of biopsy results in patients with NAFL/D found that on 40% of occasions there was a staging difference between the 2 compared biopsy samples [152].

Alternatives

Beyond liver biopsy there is increasing interest in the use of non-invasive diagnostic markers. Chapter 2 reports the findings of a systematic search of non-invasive markers of liver fibrosis.

1.4.2 Histological grading and staging in non-alcoholic fatty liver /disease

The histological determination of NAFL/D is based on three components: i) steatosis, ii) hepatocellurlar injury and inflammation, and iii) fibrosis. 'Grading' is typically used in reference to measures of ongoing disease activity (necroinflammation). 'Staging' refers to the longer term disease progression (fibrosis and parenchymal or vascular remodelling)[153].

Steatosis

By definition steatosis must be present for a diagnosis of NAFL/D. On histological examination a minimum of 5% of hepatocytes containing fat droplets must be present to meet the diagnosis.

Non-alcoholic steatohepatitis

The NAFLD Activity Score score[154] was developed by the Pathology Subcommittee of the NASH Clinical Research Network. It is the un-weighted sum of scores assigned to steatosis, ballooning and lobular inflammation and was originally developed to examine treatment response histologically (Table 1-4). Whilst it can be used to monitor NASH, it is neither linear nor designed as a diagnostic tool. In practice the sum score is often interpreted as: 0-2 unlikely NASH, 3-4 indeterminate, and ≥5 NASH likely.

Table 1-4 NAFLD Activity Score: histological criteria

	Definition	*Score*
Steatosis grade	<5%	0
	5-33%	1
	34-66%	2
	>66%	3
Lobular inflammation	No foci	0
	<2 foci per 200x field	1
	2-4 foci per 200x field	2
	>4 foci per 200x field	3
Ballooning	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2

NAFLD non-alcoholic fatty liver disease

Liver fibrosis

There are multiple liver fibrosis staging systems in use (including Scheuer[155], Ishak[156] and Metavir[157], see Appendix B) for chronic liver disease, which were primarily designed for the staging of hepatitis C induced fibrosis and cirrhosis. These are largely interchangeable with similar criteria, however more recently there has been a tendency towards the Kleiner criteria[154] developed specifically in NAFLD from the Brunt criteria[158], see Table 1-5.

Table 1-5 Kleiner (modified Brunt) non-alcoholic fatty liver disease histological fibrosis staging criteria

Stage	*Fibrosis features*
0	None
1	Perisinusoidal or periportal
1A	Mild, zone 3, perisinusoidal
1B	Moderate, zone 3, perisinusoidal
1C	Portal/periportal
2	Perisinusoidal and portal/periportal
3	Bridging fibrosis
4	Cirrhosis

1.5 Non-alcoholic fatty liver disease and type 2 diabetes

1.5.1 Prevalence

The prevalence of NAFL/D amongst people with type 2 diabetes is reported to be higher than in the general population.

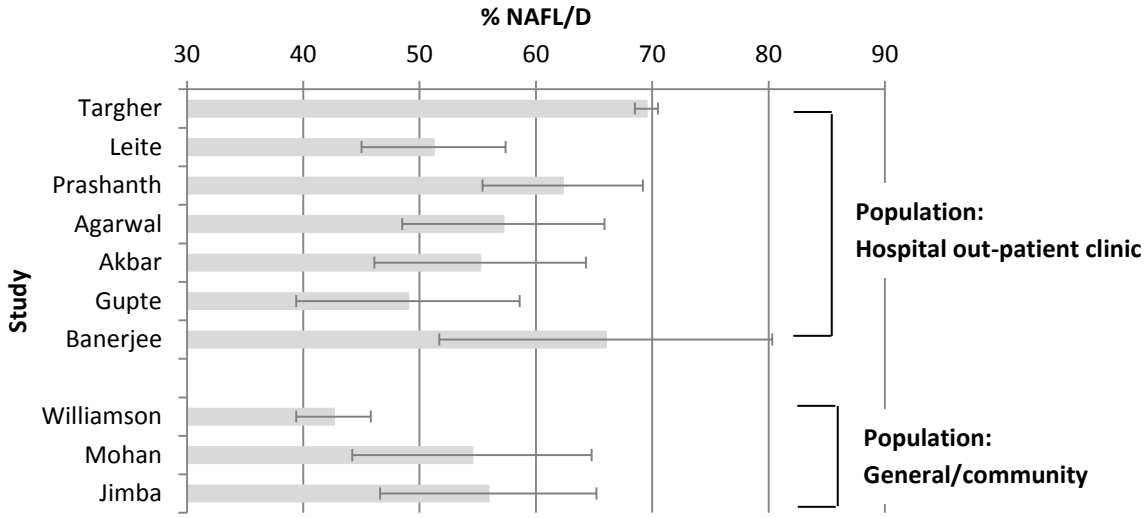
Extensive searching identified 10 studies (published prior to 31st March 2012) reporting the prevalence of NAFL amongst people with type 2 diabetes (Table 1-6)[57,159-167]. All of them based the diagnosis of NAFL on USS (with four supplemented with liver biopsy providing NASH and fibrosis prevalence), however, nearly all (8/10) were based on subjects drawn from secondary care diabetes outpatients clinics and are therefore not representative of all patients with type 2 diabetes. In addition there was wide heterogeneity in the age and sex of subjects studied.

Estimates of the prevalence on NAFL ranged from 35-70%. Of note there were two large European studies: the first including nearly 1000 UK community-based subjects (the Edinburgh Type 2 Diabetes Study) with a NAFL prevalence of 43%[165], and the second including nearly 3000 Italian hospital out-patient subjects with a higher NAFL prevalence of 70%[162].

The four biopsy studies all used hospital out-patient populations (three in India, one in Brazil). They were relatively consistent in their NASH findings, prevalence 26-36%, but there was a larger discrepancy in their fibrosis findings, prevalence 7-33%[159,161,163,164].

Overall, the estimates based on hospital out-patients (and therefore subject to selection bias) tended to have higher point estimates than the three general population studies (Figure 1-10). Given the aforementioned issues it is likely that the most plausible true prevalence of NAFL in people with type 2 diabetes is below ≤50%.

Figure 1-10 Prevalence of non-alcoholic fatty liver (+/- disease) in people with type 2 diabetes. Values are % and 95%CI.



NAFL/D non-alcoholic fatty liver +/- disease

Table 1-6 Prevalence of non-alcoholic fatty liver /disease in people with type 2 diabetes

Study	Year	Country	Population	n	Age, years (mean/median/range)	Male	Method of NAFL/D diagnosis	NAFL prevalence	NASH prevalence	Fibrosis prevalence
Targher[162]	2006	Italy	Out-patient	2839	40-86	-	USS	69.5%	-	-
Williamson[165]	2008	UK	General	939	61-76	-	USS	42.6%	-	-
Leite[163]	2007	Brazil	Out-patient	244	<65	30.0%	USS + liver biopsy	51.2%	31.3%	33.0%
Prashanth[161]	2005	India	Out-patient	204	20-70	-	USS + liver biopsy	62.3%	25.5%	15.2%
Mohan[166]	2009	India	General	132	50.4	56.1%	USS	54.5%	-	-
Agarwal[167]	2011	India	Out-patient	124	>35	59.7%	USS	57.2%	-	-
Akbar[160]	2003	Saudi	Out-patient	116	54	27.0%	USS	55.2%	-	-
Jimba[57]	2003	Japan	General	111	49	69.0%	USS	55.9%	-	-
Gupte[159]	2002	India	Out-patient	100	-	-	USS + liver biopsy	49.0%	28.0%	7.0%
Banerjee[164]	2008	India	Out-patient	47	-	-	USS + liver biopsy	66.0%	36.2%	31.9%

NAFL non-alcoholic fatty liver; **NAFL/D** non-alcoholic fatty liver disease; **NASH** non-alcoholic steatohepatitis; **USS** ultrasound scan

1.5.2 Complications

Diabetes is also associated with an increased risk of hepatic complications. In the Verona Diabetes Study chronic liver disease had an SMR of 2.52[168]. A Danish study examined the incidence of HCC amongst hospitalised patients and found it to be higher amongst people with diabetes (SMR 4.0 men and 2.1 women) [169]. Hassan et al investigated the diagnosis of diabetes in patients with HCC and found an adjusted OR of 4.3(1.9-9.9) for diabetes in HCC compared with other primary cancers [170].

1.6 Non-alcoholic fatty liver /disease and cardiovascular disease

Patients with CLD, and in particular NAFL/D, are reported to have both higher all-cause mortality[171,172] and cardiovascular (CV) mortality rates than the general population[113,173].

Two questions are currently not fully answered:

(i) Is the relationship between NAFL/D and CVD driven by their shared risk factors, or does NAFL/D 'cause' CVD independently?

(ii) Is the increased risk of CVD in people with NAFL/D present in those with simple NAFL or is more advanced NAFLD required?

1.6.1 The atherogenic liver hypothesis

Many of the likely causal explanations for both fatty liver and atherosclerosis are shared (e.g. insulin resistance, dyslipidaemia, systemic inflammation, see Table 1-7). However, the concept of the liver-vessel axis hypothesis[174] could explain the biological mechanisms linking the liver directly to the accelerated atherosclerosis proposed in NAFL/D.

Table 1-7 Possible pathophysiological associations between NAFL/D and CVD

Factor	*Atherosclerosis*	*NAFLD*
Atherogenic hyperlipidaemia	Associated with high low and very-low density lipoprotein and with low high density lipoprotein	Prevalence increased in mixed hyperlipidaemia
Arterial hypertension	Associated and partially reversible with a decrease in hypertension	Prevalence higher in people with hypertension
Hyperhomocycteinaemia	Associated	Evidence from animal and human studies, including HCV steatosis
Type 2 diabetes	Strongly associated	Prevalence increased and a risk factor for the development and progression of NAFLD
Abdominal obesity	Strongly associated	Associated and a predictor of liver fibrosis
Prothrombotic state	Association with fibrinogen, PAI-1, Factor VII, Factor VIII, platelet reactivity and others	Associated with fibrinogen, PAI-1, Factor VII, Factor VIII and decreased tissue-type plasminogen activator
Systemic inflammation	Associated with CRP and other acute-phase proteins	Postulated major determinant for the development of NAFLD
Metabolic syndrome and insulin resistance	Strongly associated	Prevalence higher in metabolic syndrome and a risk factor for the progression of NAFLD
Sex	Men < (premenopausal) women	Findings not conclusive
High-fat diet	Strong association with lifestyle	Reported in NAFLD and impaired postprandial lipid metabolism
Cigarette smoking	Strongly associated and reversible by stopping	Findings not conclusive
Antioxidants	Findings not conclusive	Findings not conclusive
Sedentariness	Independent association	Associated with NAFLD; exercise is recommended as a treatment

Adapted from Loria *et al*[174]

CRP C-reactive protein; **CVD** cardiovascular disease; **HCV** hepatitis C virus; **NAFL/D** non-alcoholic fatty liver +/- disease; **PAI-1** plasminogen activator inhibitor-1

Although links between liver disease and CVD have frequently been attributed to the inter-relationships between NAFL/D, obesity and the metabolic syndrome, it is emerging that there is an increased risk of CV events in subjects with NAFLD, independent of these traditional risk factors[175-178].

There is increasing evidence that the consequences of NASH include atherosclerosis via further insulin resistance leading to atherogenic hyperlipidaemia (low high density lipoprotein (HDL) with high TG and LDL levels) and systemic inflammation through pro-inflammatory and pro-atherogenic factors (e.g. interleukin-6 (IL6), TNFα, NF-κB)[179,180] and this is supported by the observation that cardiovascular risk is greater among patients with nonalcoholic steatohepatitis than among those with simple steatosis[113,181-183].

1.6.2 Prevalence

Several large cross-sectional studies have shown a higher prevalence of CVD in people with NAFL/D, beyond any risk conferred by the coexistence of type 2 diabetes other traditional CV risk factors. In the NHANES II cohort study over 2000 individuals were identified as having NAFLD by USS, with an OR 1.23 for prevalent CVD compared to people without NAFL/D[184]. In a large study of type 2 diabetes out-patients (n=2839), all components of CVD were more common in people with USS diagnosed NAFL/D than in those without: coronary artery disease (CAD) 27 vs 18%, cerebrovascular disease 20 vs 13% and peripheral vascular disease 15 vs 10%. This association remained independent after adjustment for multiple CV risk factors[162]. A prospective study of patients undergoing coronary angiography found that CAD was present in significantly more patients with NAFL/D (USS diagnosed) than in those without NAFL/D, 85 vs 64% and that after adjustment the association persisted with OR 2.31[185]. All of these studies used USS to diagnose NAFL/D not liver biopsy, the gold standard, however this allowed the authors to investigate community/general populations.

1.6.3 Gamma-glutamyl trasnferase and cardiovascular disease

To date, the most commonly cited hepatic marker of incident CVD and CV mortality is plasma gamma-glutamyl transferase (GGT). However, reports are conflicting, and it is unclear if any relationship relates to a specific hepatic influence on CVD or whether GGT is a surrogate marker for systemic inflammation.

Systematic searching for all publications identified 8 papers reporting the association between GGT with incident CVD (Table 1-8) and 15 with CVD mortality (Table 1-9).

Gamma-glutamyl transferase and incident cardiovascular disease

Of the eight studies investigating incident CVD, five found a statistically significant relationship[186-190] with GGT and three found no relationship[162,191,192].

The two largest studies (cohorts in excess of n=10,000) both found a statistically significant relationship between increased GGT and the incidence of CVD. For any CAD event (fatal and non-fatal) Lee et al found an incident rate of 6.3 events /1000 person-years in the highest GGT quartile compared to 4.2 events /1000 person-years in the lowest. Similarly there were significant multivariable adjusted (cardiovascular risk factors) hazard ratios for both men and women (men HR 1.27 (95%CI 1.02-1.59) , women HR 1.32 (95%CI 0.96-1.80) in the top quartile of GGT compared to the lowest quartile[186]. The study by Jousilahti[187] was limited to incident stroke only, however, still found a significant association between $\log_{10}$GGT and all types of incident stroke after extensive adjustment for CV risk factors (male HR 1.24 (95%CI 1.03–1.50), female HR 1.33 (95%CI 1.06–1.65)[187].

Two studies were limited to patients with diabetes[162,189,192] with neither finding an association between GGT and incident CVD. Note that whilst Targher found an association in the 2005 publication[189], when the same cohort was followed up for a longer time period the association was no longer statistically significant[162].

Gamma-glutamyl transferase and cardiovascular mortality

Of the 15 studies investigating CVD related mortality seven found a statistically significant association with GGT[188,190,193-197], four had mixed results[186,198-200] and four found no relationship[173,192,201,202]. Of the four with mixed relationships three found an association for males but not females[198,199,203] and the fourth found a relationship for younger patients but not older patients (age cut-off 70 years)[200].

The largest study[193] followed nearly 300,000 subjects attending hospital for the first time (in- or outpatient) in Vienna, Austria for >2,000,000 person-years. They found

increasing mortality increasing with increasing GGT for all types of CVD (vascular HR 1,7 (1,6-1,8), ischaemic heart disease HR 1,7 (1,6-1,9), cerebrovascular disease HR 1,4 (1,1-1,6) for elevated GGT (>56 U/L) vs normal levels in men, similar results for females). Despite the impressive size of this study it should be interpreted with knowledge of the ascertainment bias of hospital patients receiving GGT measurement and that there was limited adjustment of results for existing CV risk factors due to the study being retrospective and reliant on routinely collated data.

Both Monami[202] and Sluik[192] investigated patients with type 2 diabetes without prevalent CVD at baseline and found no association between GGT and CVD mortality over modest follow-up periods. Both studies adjusted extensively for known CV risk factors.

Table 1-8 Gamma-glutamyl transferase and incident cardiovascular disease

Author	Year	Country	Population	N	Male, %	Age, years[a]	Follow-up, years	Key findings
Lee[186]	2006	Finland	General	28,838	47.9	25-64	11.9	Incident CVD: 5.1% GGT and all CVD– significant relationship for both males and females (p<0.001) GGT and nonfatal MI – significant relationship for males (p=0.04) but not females
Jousilahti[187]	2000	Finland	General	14,874	48.2	25-64		Incident stroke: male 3.6%, female 2.7% GGT and stroke - significant relationship for both males and females
Lee[188]	2007	Canada	Framingham Offspring Study	3,451	48.1	44	19.1	Incident CVD: males 21.8%, females 9.7% GGT and all CVD - significant relationship (p≤0.01)
Monami[191]	2008	Italy	General, excluded existing diabetes and CVD	2,617	46.4	40-75	3.3	Incident CVD (fatal + nonfatal): 0.8% GGT and all CVD - significant relationship (p<0.001)
Targher[162]	2007	Italy	Type 2 diabetes out-patient clinic	2,103[b]	63	60	6.5	Incident CVD: fatal + nonfatal 18.3%, nonfatal 10.4% GGT and all CVD – no association after controlling for the metabolic syndrome
Targher[189]	2005	Italy	Type 2 diabetes out-patient clinic, excluded existing CVD	2,103[b]	-	-	5	Incident CVD: fatal + nonfatal 11.8%, nonfatal 8.1% GGT and all CVD – no association after controlling the metabolic syndrome
Sluik[192]	2012	Germany Netherlands	Type 2 diabetes out-patient clinic, excluded existing CVD	1,280	-	58	8.2	Incident CVD (fatal + nonfatal) 11.1% GGT and all CVD – no association
Emdin[190]	2001	Italy	CVD out-patient clinic	469	85.3%	59	2.7	Incident CVD (fatal + nonfatal)

Author	Year	Country	Population	N	Male, %	Age, years[a]	Follow-up, years	Key findings
								GGT and all CVD - significant relationship (p=0.036)

[a]values are mean, median or range; [b]same cohort of patients followed for 2 differing time periods.

CVD cardiovascular disease; **GGT** gamma-glutamyl transferase

Table 1-9 Gamma-glutamyl transferase and cardiovascular mortality

Author	*Year*	*Country*	*Population*	*N*	*Male, %*	*Age, years*[a]	*Follow-up period, years*	*Findings*
Kazemi-Shirazi[193]	2007	Austria	Hospital attenders	283,438	45.4	50	7.6	CVD mortality 6.1% GGT and all CVD/IHD/CEVD significant relationship
Ruttman[194]	2005	Austria	General	163,944	45.6	42	males 11.1 females 12.0	CVD mortality male 2.1%, female 1.6% GGT and all CVD– significant relationship for both male and female (p<0.001)
Strasak[198]	2008	Austria	General	76,113	42.5	42	males 9.8 females 10.6	CVD mortality male 3.0% female 2.2% GGT and all CVD/CHD/stroke – significant relationship for male (p<0.001) but not female
Lee[186]	2006	Finland	General	28,838	47.9	25-64	11.9	CVD mortality 2.1% GGT and all CVD– significant relationship for males (p<0.01) but not female
Ruhl[201]	2009	USA	General	11,630	47	>2months	8.8	CVD mortality 4.1% GGT and all CVD – no association
Wannamethee[195]	1995	UK	British Regional Heart Study - General	7,613	100	40-59	11.5 [Excl. deaths in first 5 years]	CVD mortality 5.9% GGT and ischaemic heart disease– significant relationship after adjustment for pre-existing ischaemic heart disease
Hozawa[196]	2007	Japan	General	6,846	39.8	>30		CVD mortality male 3.0%, female 1.2% GGT and all CVD– significant relationship for both male and female

Author	*Year*	*Country*	*Population*	*N*	*Male, %*	*Age, years*[a]	*Follow-up period, years*	*Findings*
Haring[199]	2009	Germany	General	4,160	49.1	50.6	7.2	GGT and all CVD– significant relationship for male (p<0.001) but not female
Lee[188]	2007	Canada	Framingham Offspring Study	3,451	48.1	44	19.1	CVD mortality male 13.9%, female 7.3% GGT and all CVD– significant relationship (p≤0.001)
Calori[173]	2011	Italy	General	2,011	44.0	57	15	GGT and all CVD – no association
Monami[202]	2007	Italy	Type 2 diabetes out-patient clinic, excluded existing CVD	1,952	51.6	65.6	6.4	CVD mortality 7.8% GGT and all CVD – no association after extensive adjustment
Sluik[192]	2012	Germany Netherlands	Type 2 diabetes out-patient clinic, excluded existing CVD	1,280	-	-	8.2	CVD mortality 2.7% GGT and all CVD – no association after extensive adjustment
Emdin[197]	2009	Italy	Cardiac catheterisation patients	474	80.4	64	1.5	CVD mortality 5.5% GGT and all CVD– significant relationship
Emdin[190]	2001	Italy	Ischaemic heart disease patients	469	85.3	59	2.7	CVD mortality 6.0% GGT and all CVD– significant relationship (p<0.001)
Lee[200]	2009	USA	Minnesota Heart Survey - General	386	56.5	68	10-12	GGT and all CVD– significant relationship for age<70yrs (p=0.02) but not >70yrs

[a]values are mean, median or range.

CHAPTER 2 Systematic literature review: non-invasive markers of liver fibrosis in non-alcoholic fatty liver disease

This systematic review will follow the format of a Cochrane Review of diagnostic test accuracy (http://srdta.cochrane.org/handbook-dta-reviews). The protocol, study selection and data extraction forms are available in Appendices C and D. The original intention had been to have two reviewers, as is standard for systematic reviewing and as described in the protocol, however due to unforeseen circumstances the second reviewer was unable to complete the review. A summary PRISMA statement checklist is included in Appendix E.

2.1 Background

2.1.1 Role of non-invasive markers of liver fibrosis

NAFL/D involves a wide spectrum of disease with histological findings ranging from fat deposition only, through inflammatory changes to fibrosis and cirrhosis[204]. Clinical difficulties arise as most patients with NAFL/D have no clinical signs or symptoms until the disease has progressed to significant fibrosis, with a proportion progressing to end-stage liver disease[205,206].

Confirmation of liver fibrosis in NAFLD is currently only definitive using liver biopsy[158]. This is an invasive investigation requiring a 6 hour inpatient observation period post procedure. Common minor complications include pain, nausea, and vomiting. Potential significant complications include haemorrhage, biliary leakage and transient jaundice, with a small but acknowledged mortality rate of <0.5%[150,207]. It is therefore not feasible to use liver biopsy recurrently for follow-up investigation of patients or in large population based research studies given its unfavourable nature.

As a result, over recent years, alternative non-invasive tools for the diagnosis of liver fibrosis have been developed. In addition, to the problems noted above, sometimes the staging system cannot provide a dynamic picture of the changes that have already taken place in the liver. Therefore, alternative biological parameters may be considered to be more accurate than liver biopsy in reflecting the fibrogenesis in hepatocytes. It is unclear if any of these non-invasive tools have any advantage over others, in terms of diagnostic test accuracy and practicality of application.

2.1.2 Selected liver fibrosis markers

Many liver fibrosis markers have been investigated, some more so than others. This section will provide an overview of some of the most commonly studied/used fibrosis markers in NAFLD.

Because liver fibrosis is characterised by excess collagen deposition, decreased extracellular matrix degradation and activation of hepatic stellate cells most serum markers of hepatic fibrosis are related to this common pathway. A number of individual markers have been investigated, before later being combined into supposedly more accurate panel markers. Imaging modalities focus on the architectural changes present in fibrotic liver.

In general these markers were first investigated in chronic viral hepatitis e.g. aspartate aminotransferase (AST) -to-platelet ratio index (APRI)[208], the Forns test[209], FibroTest (Biopredictive SAS, Paris, France)[210-216] and transient ultrasound elastography (TUE, FibroScan, Echosens, Paris, France)[217].

Individual markers

Hepatocyte apoptosis

CK18 is a marker of hepatocyte apoptosis, a process occurring in the presence of hepatocyte damage and therefore as part of the fibrotic pathway. As part of the apoptotic process within the liver effector caspases (which act on a number of different substrates inside the cell) are activated. As a result of this one of the

substances generated is CK18[218,219]. More frequently CK18 has been described as a marker of NASH[82,84,220,221] than for the diagnosis of liver fibrosis.

Extracellular matrix proteins and formation of fibrosis

Type IV collagen (COL-IV), hyaluronic acid (HA) and laminin are all extra-cellular matrix proteins[222,223]. All are produced by connective tissue cells and are maintained by the balance of production, with degradation by liver sinusoidal endothelial cells[224,225]. It is understandably hypothesised that extracellular-matrix proteins could be used as a markers of liver function and fibrosis, with reduced hepatic function being associated with increased levels of such markers.

Metalloproteinases are a group of peptidases involved in degradation of the extracellular matrix and therefore tissue inhibitor metalloproteinase-1 (TIMP-1) levels are thought to be related to hepatic fibrosis levels. In addition, TIMP-1 promotes cell proliferation in a number of cell types including the liver.

YKL-40 is an inflammatory glycoprotein mainly produced by macrophages, neutrophils, and vascular smooth muscle cells[226] and involved in endothelial dysfunction by promoting chemotaxis, cell attachment and migration, reorganization, and remodelling of the extracellular matrix as a response to endothelial damage. YKL-40 has roles in cell proliferation and differentiation, angiogenesis, inflammation, remodelling of the extracellular matrix, and the innate immune response[227].

Panel markers

Aspartate aminotransferase to platelet ratio index

APRI was developed in a cohort of 192 patients with hepatitis C virus[208]. The original study determined a cut-off of 1.5 for significant fibrosis (Ishak stages 3-6) and 2 for cirrhosis (Ishak stages 5-6).

$$APRI = [(AST\ (U/L) / ULN\ (U/L)) / platelet\ (x10^9/L)]*100$$

A recent study 145 patients with biopsy proven NAFLD using a cut-off of 1 for advanced fibrosis had an unimpressive AUROC of 0.67 (0.54-0.8) with an associated

sensitivity of 27% and specificity of 89% at the most accurate cut-off[228]. A further small study of 30 NAFLD patients had similar findings with significant fibrosis AUROC 0.56 (0.35-0.78) and cirrhosis AUROC 0.57 (0.19-0.95)[229].

Aspartate aminotransferase to alanine aminotransferase ratio

The aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio is a simply calculated marker using two commonly measured liver enzymes. Originally an elevated AST and AST:ALT ratio was thought to be related to a high alcohol intake prior to the realisation that some heavy drinkers did not have any elevation and that the elevation related to the resulting liver damage[230,231].

The difficulty with the interpretation of the AST:ALT ratio lies with differing liver disease aetiologies. Typically ALD leads to an elevated ration (often >2) with NAFLD and hepatitis C virus having a ratio ≤1. This is further complicated by the co-existence of the metabolic syndrome and alcohol excess in many patients. A recent biopsy based study of 140 participants with NASH or ALD found mean ratios of 0.9 and 2.6 respectively[232]. As the stage of NASH fibrosis increased, so did the AST:ALT ratio with the mean ratio in NASH cirrhosis being 1.4, although there were only five patients in this group.

BAAT

BAAT is a system based on BMI, age, ALT and TG.

> *It is scored as follows: BMI ≥28 score 1, age ≥50 score 1, ALT ≥2x upper normal limit score 1, and TG ≥1.7mmol/l score 1, with total score being 0-4.*

Ratziu and colleagues[119] developed this simple scoring system in a cohort of 93 obese patients (30% with fibrosis) in an attempt to derive a simple approach to the diagnosis of NASH fibrosis without the need for biopsy in an increasingly obese population. It is good for predicting those that do not have severe fibrosis (negative predictive value, NPV), but only 45% of those with a positive test (positive predictive value, PPV) actually have severe fibrosis (using a cut-off of 1) so further investigation would be required[119].

BARD

BARD is a system based on BMI, AST:ALT ratio and a diagnosis of diabetes.

> *Scoring is as follows: BMI ≥28 score 1, ALT:AST ratio ≥0.8 score 2, and diagnosis of diabetes score 1.*

Again the NPV appears much better than PPV for severe fibrosis, with only 27-43% of those with a positive test actually having severe fibrosis, thus not completely averting the need for biopsy[233]. It was also trialled in a Japanese cohort where it was found to be less effective [234].

Enhanced Liver Fibrosis panel

The Enhanced Liver Fibrosis (ELF) panel includes: TIMP 1, HA, and aminoterminal peptide of pro-collagen III (P3NP). These are combined into the following formula:

> *ELF = -7.412 + (ln(HA)*0.681) + (ln(P3NP)*0.775) + (ln(TIMP1)*0.494)*

Prior to a NAFLD specific validation study, the formula was derived using a cohort of 921 patients attending for liver biopsy in an international multi-centre study and evaluated for the ability to discriminate between biopsy stages using a wide range of potential markers [235]. The results for the ELF formula resulted in the maximum separation of the biopsy groups over the full range of fibrosis stages. It did however note variety in the performance of the test for different aetiologies of CLD with a wide range represented in the study (majority chronic hepatitis C virus (n=496), ALD, fatty liver, hepatitis C virus, primary biliary cirrhosis, autoimmune hepatitis, haemochromatosis, cryptogenic cirrhosis and granulomatous disease). Since then a further validation study in NAFLD has been performed comparing ELF with the NAFLD Fibrosis Score (NFS) and the NFS in combination with ELF. In this well conducted study of nearly 200 individuals with biopsy confirmed NAFLD they found ELF to be superior to the simple clinical model (modified NFS), with the combined formula showing slight further improvement[236].

Fibrosis-4 Index

Using 270 consecutive patients with treatment naïve hepatitis C virus Wai and colleagues[208] developed a marker (Fibrosis-4 Index, FIB4) using two regularly measured laboratory parameters.

$$FIB4 = (age[years] * AST[U/L]) / (platelets[x10^9/L] * \sqrt{ALT\ [U/L]})$$

A large number of measures were investigated with platelet count, AST and alkaline phosphatase being the variables in the best models for prediction of significant fibrosis. However, models with only platelet count and AST level were deemed more simple and had accuracies comparable with those with three or more variables (AUROC platelets, AST and alkaline phosphatase 0.82 (0.76-0.88) and AUROC APRI 0.80 (0.74-0.87)).

Fibrometer

Fibrometer NAFLD includes: glucose, AST, ferritin, platelet, ALT, body weight and age.

*Fibrometer NAFLD = 0.4184 *glucose (mmol/l) + 0.0701*AST (UI/l) + 0.0008 *ferritin (lg/l) - 0.0102* platelet (G/l) - 0.0260*ALT (UI/l) + 0.0459*body weight (kg) + 0.0842*age (yr) + 11.6226.*

The exact formula varies by underlying CLD aetiology and was first developed for viral hepatitis and ALD[237]. For use in NAFLD it was developed in a group of 235 patients with NAFLD [238] with less than three months between biopsy and blood sampling and had an overall accuracy (compared to liver biopsy) of 91.1% (95% CI 87.4-94.7%).

FibroTest

FibroTest includes: α_2-macroglobulin, apolipoprotein A1, haptoglobin, γ-glutamyl transpeptidase and total bilirubin. The score ranges from 0 (reflecting to no fibrosis) to 1 (reflecting cirrhosis)[239].

$$FibroTest = 4.467*\log_{10}alpha2macroglobulin(g/L) - 1.357*\log_{10}haptoglobin(g/L) + 1.017*\log_{10}GGT(U/L) + 0.0281*age(years) + 1.737*\log_{10}bilirubin(micromole/L) - 1.184*ApoA1(g/L) + 0.301*sex(female=0, male=1) - 5.54$$

A systematic review[240] searching Feb 2001 to June 2008 identified 2 studies for FibroTest in NAFLD[241]. For both groups the lower cut-off value yielded high sensitivity and moderate specificity i.e. those scoring above the threshold probably do have advanced fibrosis or cirrhosis, but a negative result is not so reassuring. For the higher cut-off there were low sensitivities and high specificities, i.e. those with negative results can be reassured but those with positive results may need further investigation.

FibroTest produced non-interpretable results in about 5% of cases. Causes of failure included false negative results due to high haptoglobin measures in cases of acute inflammation or sepsis, and false positives with low haptoglobin measures in cases with haemolysis and high bilirubin in cases with haemolysis or Gilbert disease[239]. It has also been trialled specifically in patients with diabetes[242].

NAFLD fibrosis score

The NFS uses: AST:ALT ratio, platelet, albumin, impaired fasting glucose/diabetes, age, and BMI.

$$NFS = -1.675 + 0.037[age(yrs)] + 0.094[BMI(kg/m^2)] + 1.13[IFG/diabetes(yes=1, no=0)] + 0.99[AST/ALT\ ratio] - 0.013[platelet(x10^9/l)] - 0.66[albumin(g/dl)]$$

Developed in a cohort of over 700 biopsy confirmed cases of NAFLD the laboratory measures were collected on the same day as the liver biopsy. The agreement between the validation and estimation cohorts was evident but limited (AUROC 95%CI's 0.85-0.92 and 0.76-0.88 respectively). Two cut-off values were identified in order to confidently diagnose the presence or absence of severe fibrosis with high PPV and NPV's respectively and varying the prevalence of severe fibrosis maintained satisfactory discriminatory ability. However, approximately 25% of

patients fall between the 2 cut-offs and be termed ‘indeterminate’ and requiring liver biopsy[243].

The discriminatory ability of the NFS fell when examined in a morbidly obese cohort[244]. The number of ‘indeterminate’ patients rose to 47% of patients. This may well be due to influence of BMI and diabetes status in the formula. However, the accuracy for the diagnosis of fibrosis above the higher cut-off was still 88% and below the cut-off fibrosis excluded with 98% certainty.

Imaging

Elastography

Elastography allows the measurement of stiffness of human tissues by measuring the velocity of wave propagation. The theory behind elastogaphy measures is that scarred and fibrotic tissue is stiffer than normal hepatic tissue and that this can be detected. Elastography is currently available in two forms: TUE which is 1-dimensional, and magnetic resonance elastography (MRE) that can be 2- or 3-dimensional. A phantom study found excellent correlation between TUE and MRE (r^2=0.93)[245].In addition to being non-invasive, a key benefit of elastography over liver biopsy is the ability to image/sample a larger proportion of the liver: liver biopsy samples 1/50000 of the liver, TUE 1/10000 and MRE potentially the whole liver.

One dimensional TUE involves the use of an ultrasound transducer at the end of a vibrating piston. The piston produces a low amplitude and frequency vibration that generates a sheer wave that passes through the liver. The ultrasound then detects the sheer wave through the liver and measures its velocity. The liver stiffness measure (LSM) is expressed in kilopascals. The harder the tissue the faster the sheer wave travels. TUE measures a cylinder 1cm wide by 4cm long, between 2.5cm and 6.5cm below the skin surface.

The standard ‘M’ probe is placed in the intercostal space overlying the liver with the patient in the supine position. Using ultrasound to guide the positioning a portion of the liver that is at least 6cm thick and free from large vessels is used for the

investigation. Use of TUE is therefore contraindicated in people with known structural liver abnormalities or masses (tumours or cysts). In addition, for reasons that are unclear its use is contraindicated in people with a pacemaker.

Ten LSM are recorded by the machine (taking approximately 3-10 minutes).and the median calculated. In order to be considered valid at least 60% of the LSM need to be successful, and the inter-quartile range <30% of the final (median) result.

Success acquisition rates of 89.8%[246] and 95.1%[247] have been reported for patients with NAFLD. The main factor influencing the acquisition of valid readings is obesity with the failure rate in obese patients (BMI >30) 25.5% vs 2.2% in those with lower BMI[246] . In order to try and address this issue the manufacturers of Fibroscan have developed a newer probe (the 'XL' probe) for use in larger patients. Preliminary published data for the XL probe using patients with BMI >30 has found a 60% increase in the success rate compared with the standard M probe (76.9% vs 45.5%)[248]. Probe details are given in Table 2-1.

Table 2-1 Fibroscan probe details

	'M' probe	***'XL' probe***
Central ultrasound frequency	5 MHz	3.5 MHz
Ultrasound transducer focal length	35 mm	50 mm
Probe tip external diameter	9 mm	12 mm
Vibration amplitude	2 mm	3 mm
Measurement depth	25-65 mm	35-75 mm

In studies of reproducibility of TUE in people with CLD inter-observer agreement was 0.98 (95% CI 0.977, 0.987)[249]. Gender, age, aetiology and liver disease severity did not affect inter-observer variability. High BMI, increased steatosis on USS and histology, and low stage fibrosis were associated with reduced inter-observer agreement. In the same study the intra-observer agreement was 0.98. Over time and with practice, reproducibility of results improved (month 1 0.97, month 2 0.99, month 4 0.99), with maximal agreement reached within 2 months of regular use[249].

MRE can be implemented on conventional magnetic resonance imaging (MRI) equipment. In addition a sheer wave source and specialised software are required. Sheer waves are generated by an external device at frequencies between 40 and 200Hz. Sheer waves can be generated by electromechanical voice coils, passive rigid rod drivers and most commonly passive pneumatic drum drivers. The passive pneumatic driver has several advantages over the others. It can be easily placed against the body and can be manoeuvred into any orientation.

The software required for MRE includes a specialised phase-contrast magnetic resonance pulse sequence to image the waves and an inversion algorithm to process them

Benefits of MRE over TUE include[250]: a freely orientated field of view; no acoustic window requirement; operator independence; insensitivity to body habitus; potential assessment of the entire liver parenchyma; ability to obtain conventional magnetic resonance imaging at the same time

Obesity currently limits the use of TUE, despite the newer 'XL' probe. Yin et al[251] found that the physical application of the external sheer wave generator to larger patients was not problematic and that steatosis did not affect liver stiffness measurements using MRE.

The main limitations of MRE are: cost, body habitus limiting access through the scanner and scan acquisition time. The two main methods of image generation are spin-echo ad echo-planar. One of the difficulties with 3-dimensional spin-echo imaging is the long acquisition time. Single shot echo-planar sequencing is much faster. Huwart et al[252] compared the two methods and found that echo-planar imaging substantially reduced the examination time from 20 down to 4 minutes without reducing image quality or diagnostic ability.

A number of studies have measured MRE of the liver in healthy volunteers and patients. The patients include a wide range of aetiologies of liver disease, but the majority are hepatitis B virus and hepatitis C virus. Average "normal" readings amongst healthy controls range from 2.0 to 3.1. Reporting of values for different

stages of fibrosis varied between papers, with some reporting average values and other diagnostic cut-offs. Reliability of MRE was examined in 5 healthy volunteers and the coefficient of variation for elasticity was only 9%[253]. The reproducibility of MRE in fibrotic liver has not been reported.

2.2 Aim

To systematically identify all published original research studies examining the use of non-invasive methods for the diagnosis of liver fibrosis in patients with NAFLD

2.2.1 Review question (PICO format)

For adults with NAFL/D, are non-invasive methods as accurate (ie with equal or better sensitivity and specificity) as liver biopsy, the current gold standard, for diagnosing liver fibrosis?

2.3 Methods

2.3.1 Search strategy

Search sources:

- An electronic database search was conducted from inception to 31st March 2012; MEDLINE, EMBASE. Global Health, Web of Science, SCIRUS and Cochrane.
- Additional studies were identified via manual review of the reference lists of identified studies, review articles and citation searching using Web of Science Citation Index.
- From relevant websites: American Association for the Study of the Liver, European Association for the Study of the Liver.

Search terms employed are detailed below (Table 2-2). MESH terms were used where available.

Table 2-2 Medline search strategy

1. *Hyperlipidemia/ or *Hypertriglyceridemia/ or *Metabolic Syndrome X/ or *Obesity/ or *Insulin Resistance/ or *Diabetes Mellitus, Type 2/ or metabolic syndrome.mp.
2. fatty liver.mp. or exp Fatty Liver/
3. NAFLD.mp.
4. NASH.mp.
5. steatohepatitis.mp.
6. steatosis.mp.
7. non-alcoholic.mp.
8. 1 or 2 or 3 or 4 or 5 or 6 or 7
9. fibrosis.mp.
10. cirrhosis.mp.
11. $hepatitis.mp.
12. steatosis.mp. [mp=title, original title, abstract, name of substance word, subject heading word]
13. inflammation.mp.
14. 9 or 10 or 11 or 12 or 13
15. serum markers.mp. or exp Biological Markers/
16. diagnosis/ or "diagnostic techniques and procedures"/ or "laboratory techniques and procedures"/
17. $invasive.mp.
18. exp Liver Function Tests/
19. exp DIAGNOSTIC IMAGING/
20. ELASTOGRAPHY.MP
21. predict$.mp.
22. marker$.mp.
23. surrogate.mp.
24. 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 OR 23
25. 8 and 14 and 24
26. limit 25 to (humans and english language)
27. limit 26 to "all adult (19 plus years)"

2.3.2 Criteria for inclusion

Types of study

There was no restriction on study design. Primary studies were sort where available from identified systematic reviews and meta-analyses. Studies were required to be written in English as translation was not available for this literature review. Studies reporting AUROC analysis were included.

Subjects

All study populations allowing data on patients with NAFLD to be extracted were included. Studies were required to have ≥30 adult (age ≥18 years) subjects.

Definition of NAFLD

NAFLD was required to be defined as a biopsy diagnosis with a statement regarding the attempt to exclude other causes of liver disease (e.g. alcoholic liver disease, viral hepatitis, autoimmune disorders, metabolic disorders). Studies of obese populations without mention of NAFLD, in whom there had been a clear attempt to exclude other causes of liver disease as above, were included.

Non-invasive methods of diagnosing liver fibrosis

- Individual serum markers; or
- Marker panels (≥2 components); or
- Any imaging modality
- Any other diagnostic modality

Individual physiological markers were excluded (e.g. age, sex).

Target condition

Liver fibrosis as defined using any recognised histological classification. Fibrosis was then further sub-divided into any Metavir F1-4, moderate Metavir F2-4 and significant Metavir F3-4. Cirrhosis (Metavir F4) only was excluded.

Reference standard

Liver biopsy (any method) was the reference standard

2.3.3 Data collection and analysis

Selection of studies

One researcher (JRM) undertook all searching. Following electronic searching results from multiple databases were combined before duplicate entries were removed. Titles were then screened for relevance and where necessary abstracts reviewed. Full papers were then reviewed to confirm studies meeting the inclusion criteria. Reference lists were then scrutinised and addition studies identified from citation searching. Where a conference abstract was identified later publication of a full original article was sought.

Data extraction and management

Data extraction was undertaken by one reviewer (JRM).

The following data was extracted where available: i) study characteristics - year of study/publication, number of subjects, mean/median age, % male, % diabetes, study population and selection, NAFLD criteria, histological classification, and ii) diagnostic test characteristics – marker name, marker components/formula, AUROC, prevalence of fibrosis, marker cut-off and associated sensitivity, specificity.

Where more than one cut-off and associated sensitivity and specificity were provided the preferential figure for inclusion in this review was a) a cut-off analysed to be the most accurate, then b) a cut-off consistent with other studies/a generally accepted cut-off, and then c) cut-offs associated with a 90% sensitivity and specificity.

Methodological quality

Methodological quality assessment of the identified studies was undertaken using the risk of bias component of the Quality Assessment of Diagnostic Accuracy Studies II tool[254-256]. The Quality Assessment of Diagnostic Accuracy Studies II tool examines four domains with potential for risk of bias: i) Could the selection of patients have introduced bias?; ii) Could the conduct or interpretation of the index test have introduced bias?; iii) Could the reference standard, its conduct, or its interpretation have introduced bias?; and iv) Could the patient flow have introduced bias?

The studies identified with a definite or unclear risk of bias were not excluded from the review, but the risk taken into consideration in the interpretation of results.

Statistical analysis and data synthesis

A narrative description of the findings was compiled.

To allow across test comparisons to be made, where a sensitivity and specificity were available 2x2 tables were constructed to calculate the PPV and NPV for a hypothetical population of 1000 individuals with a) a fibrosis prevalence of 5%, and b) a fibrosis prevalence of 30%. These figures were chosen to mirror the potential fibrosis prevalence in a general population.

2.4 Results

2.4.1 Search results

Searching identified 73 potential studies for inclusion. After examination of the full text 16 studies were excluded [244,248,257-270] and are detailed in Appendix C with 57 studies included.

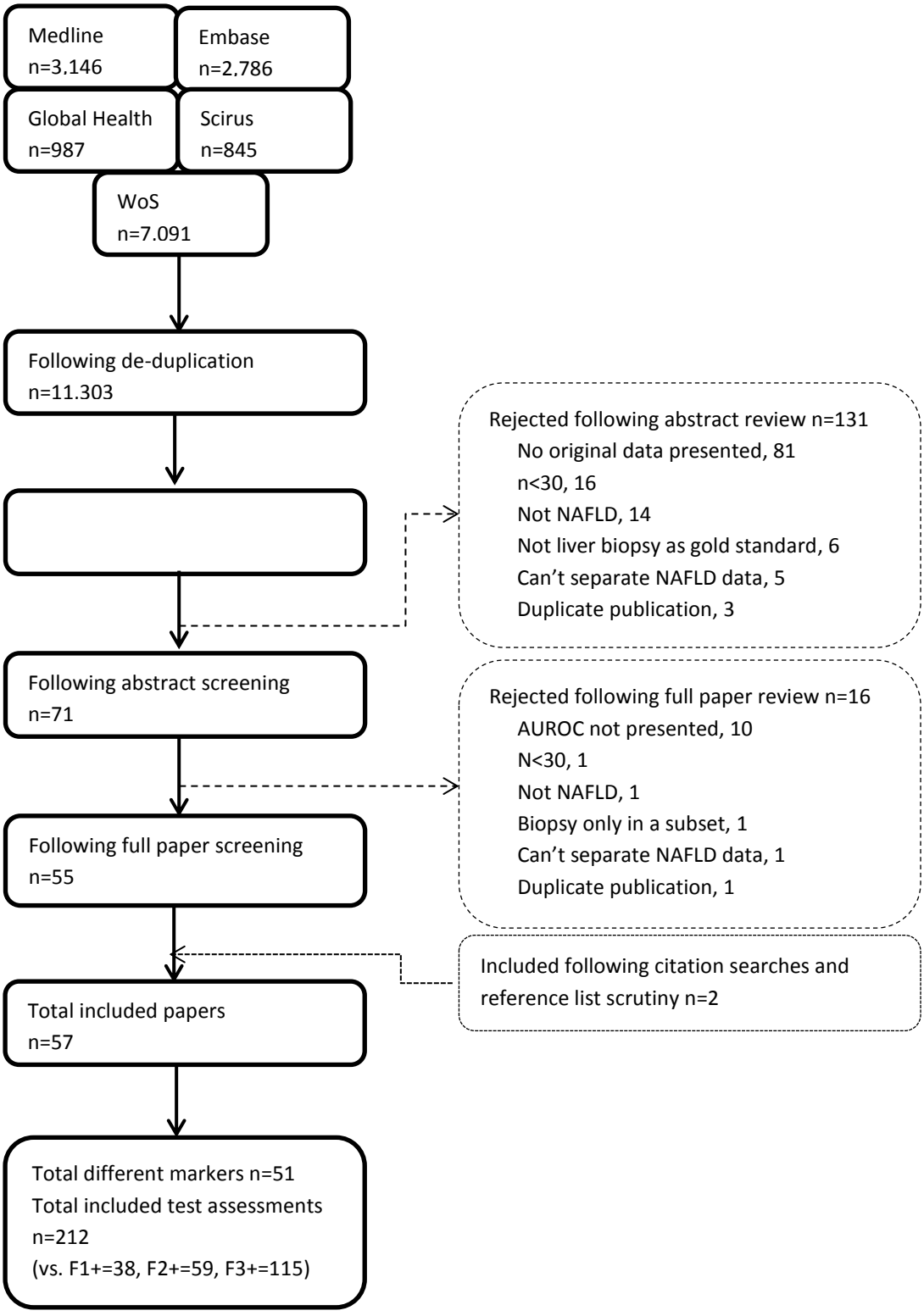
From the 57 included studies 49 different diagnostic tests were found, totalling 215 assessments of a diagnostic test (Figure 2-1) as a number of studies examined a number of different tests at each different fibrosis level. There were 10 different single markers (29 assessments), 36 panel markers (146 assessments), 3 imaging modalities (40 assessments) and 1 other marker (1 assessment) identified.

Table 2-3 shows the range of markers included.

Table 2-4 summarises the characteristics of included studies.

Table 2-5 contains a summary of panel marker components and their associated calculation formulae.

Figure 2-1 Systematic review search results



AUROC area under the receiver operating curve; **NAFLD** non-alcoholic fatty liver disease.

Table 2-3 Number of studies for each diagnostic test for liver fibrosis in NAFLD

Single markers					*Panel markers*									*Imaging + other*				
	F1	**F2**	**F3**	**Total**		**F1**	**F2**	**F3**			**F1**	**F2**	**F3**	**Total**		**F1**	**F2**	**F3**	**Total**
ALT	1			*1*	**APRI**	2	7	11	*20*	**HAIR**		2	2	*4*	**ARFI**			2	*2*
AST	2	1		*3*	**AST:ALT ratio**	1	2	9	*12*	**Hepascore**		1	1	*2*	**MRE**		1		*1*
CK18	1		1	*2*	**AP ratio**			2	*2*	**Mansousou-1**		2		*2*	**TUE**	5	15	16	*36*
HA	4	1	6	*11*	**BAAT**		2	2	*4*	**Mansousou-2**		1		*1*	**C-caffeine breath test**		1		*1*
Laminin	2			*2*	**BARD**		2	9	*11*	**NAFIC**		1	1	*2*					
SPEA	1			*1*	**CDS**		1	2	*3*	**NFS**	2	5	17	*24*					
TGF-B	-		1	*1*	**dosSantos-1**	1			*1*	**N-score**		1	2	*3*					
TIMP-1	1		1	*2*	**dosSantos-2**	1			*1*	**OELF**	1		2	*3*					
COL-IV	1		3	*4*	**dosSantos-3**	1			*1*	**P2/MS**			1	*1*					
YKL 40	1		1	*2*	**ELF**	3	2	3	*8*	**PAF**			1	*1*					
					ELF+NFS	1	1	1	*3*	**Susuki-1**			1	*1*					
					FibroMeter		1	1	*2*	**Susuki-2**			1	*1*					
					FIBROspect II		1		*1*	**Tetri-1**	1		1	*2*					
					FibroTest		4	4	*8*	**Tetri-2**	1		1	*2*					
					FIB4		1	5	*6*	**Tetri-3**	1		1	*2*					
					FM8		1	1	*2*	**Tetri-4**	1		1	*2*					
					Gholam	1	1	1	*3*	**Younossi-1**	1			*1*					
					GUCI		1	2	*3*	**Younossi-2**			1	*1*					
Totals	*14*	*2*	*13*								*19*	*40*	*87*			*5*	*17*	*18*	

APRI aspartate to platelet ratio index; **ALT** alanine aminotransferase; **ARFI** acoustic radiation force impulse; **AST** aspartate aminotransferase; **CDS** cirrhosis discriminant score; **CK18** cytokeratin-18; **COL-IV** Type IV collagen; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; **GUCI** Goteburg University Cirrhosis Index; **HA** hyaluronic acid; **MRE** magnetic resonance elastography; **NAFLD** non-alcoholic fatty liver disease; **NAS** NAFLD Activity Score; **NFS** NAFLD Fibrosis Score; **N-score** Nippon-score; **OELF** Original European Fibrosis panel; **PAF** Probability of Advanced Fibrosis; **SPEA** serum prolidase enzyme activity; **TIMP-1** metallopeptidase inhibitor 1; **TGF-β** transforming growth factor beta; **TUE** transient ultrasound elastography (Fibroscan).

Table 2-4 Included studies

	Study	*Country*	*Date*	*N*	*Population*	*Age, years*	*Men, %*	*DM, %*	*Histology*	*NAFLD[1]*	*Risk of bias[2]*
APRI, FibroTest, Hepascore	Adams[2713]	Australia	2008[3]	119	-	48.7	54.0	-	Kleiner	-	Unclear
NFS	Angulo[243]	Australia, Italy, UK, USA	2000-03	480[e] 253[v]	Secondary care. Elevated liver enzymes	47.7 47.7	55.6 48.6	28.8 32.0	Kleiner	A B C D E	None
APRI, FibroMeter, NFS	Cales[272]	France	2001-06	235	Secondary care. Two centres. Elevated liver enzymes or USS steatosis and at least one component of MS.	51.1	74.5	24.1	Metavir	A	None
TUE	de Ledinghen[273]	France, HK	2009[3]	208	Secondary care. Multicentre. Routine biopsy attendees.	51.0	55.3	-	Brunt Kleiner	-	Unclear
AST, HA, Laminin, Type IV collagen, dosSantos(1-3)	Dos Santos[274]	Brasil	2005[3]	30	Secondary care. BMI>25, USS steatosis and elevated liver enzymes.	44.9	60.0	23.3	Brunt	A B E	Unclear
TUE	Freidrich-Rust[275]	Germany	2008-09	50	Secondary care. Routine biopsy attendees.	44.0	54.0	-	Kleiner	A B C	Risk
BARD, NFS	Fujii[234 #]	Japan	2009[3]	122	Secondary care. Routine biopsy attendees.	-	38.5	36.9	Brunt	-	Unclear
TUE	Gaia[276]	Italy	2007-09	72	Secondary care. Routine biopsy attendees.	48	72.2	-	Brunt	A B	Risk
Gholam(1)	Gholam[277]	USA	2007[3]	97	Secondary care. BMI ≥40 kg/m^2 undergoing bariatric surgery.	39	16.5	23.7	Brunt	A B E	Risk

	Study	*Country*	*Date*	*N*	*Population*	*Age, years*	*Men, %*	*DM, %*	*Histology*	*NAFLD*[1]	*Risk of bias*[2]
AST, FIBROspect II	Guajardo-Salinas[278]	USA	2005-06	92	Secondary care. BMI ≥40 kg/m^2 undergoing bariatric surgery. Retrospective.	45.5	19.4	-	Kleiner Brunt	-	Risk
ELF, NFS, ELF+NFS	Guha[236]	UK	2008[3]	192	Secondary care. Two centres. Routine biopsy attendees.	48.7	64.1	-	Kleiner	A B C D E	None
BARD	Harrison[233]	USA	2001-05	827	Secondary care. Two centres. Routine biopsy attendees. Military.	49	49.0	35.0	Brunt Kleiner	A B C D	Unclear
AST:ALT ratio, BARD	Kallwitz[279]	USA	2009[3]	185	Secondary care. Obese patients undergoing bariatric surgery.	-	-	-	Kleiner	-	Risk
BAAT, HA, NFS, Type IV collagen	Kaneda[280]	Japan	1993-2004	148	Secondary care. Single centre. Routine biopsy attendees.	52.0	52.7	27.7	Brunt	A B C D E	Unclear
ALT, AST, AST:ALT ratio, SPEA	Kayadibi[281]	Turkey	2009[3]	91	Secondary care. Single centre.	-	27.5	-	Brunt	A B C E	Unclear
APRI, AST:ALT ratio, TUE	Kelleher[282 $]	France USA	2006[3]	129	Secondary care. Three centres.	53.5	-	24.8	Brunt	A B C	Risk
AST:ALT ratio, APRI, NFS	Kruger[283]	SA	2011[3]	111	Secondary care. Three centres.	52	27.0	43.2	-	A	Unclear
FibroTest	Lassailly[284]	France	1994-2011	288	Secondary care. BMI ≥40 kg/m^2 undergoing bariatric surgery.	41.6	23.6	32.0	Kleiner	A B E	Risk

	Study	*Country*	*Date*	*N*	*Population*	*Age, years*	*Men, %*	*DM, %*	*Histology*	*NAFLD*[1]	*Risk of bias*[2]
APRI	Loaeza[229]	Mexico	2008[3]	30	-	-	-	-	Metavir	-	Unclear
TUE	Lupsor[285]	Netherlands	2007-09	65	Secondary care.	41	73.8	-	Brunt	A B C E	Risk
HA	Lydatakis[286]	Greece	2000-04	50	Secondary care. Routine biopsy attendees. BMI >25	54.0	51.5	44.0	Matteoni Brunt	-	Risk
CK18, HA, NAS, TIMP-1, YKL-40	Malik[86]	USA	2003-06	95	Secondary care. Single centre. Routine biopsy attendees. Prospective.	48.5	61.1	27.4	Kleiner	A B C .	Unclear
Manousou(1,2)	Manousou[287]	UK	2011[3]	111	Retrospective database search of liver biopsies with the keywords 'steatosis' and/or 'steatohepatitis'. Normal thyroid function and not on thyroxine.	52.6	64.0	57.7	Kleiner	A B C D E	Unclear
AST:ALT ratio, APRI, BARD, FIB4, NFS	McPherson[228]	UK	2003-09	145	Secondary care. 'Fatty liver' clinic.	51	60.7	50.3	Kleiner	A F	Risk
BARD, FIB4, NFS, PAF	Miao[288$]	USA	2010[3]	686	Secondary care. Morbidly obese.	-	-	-	-	-	Risk
TUE	Miette[289]	France	2007[3]	46[e] 67[v]	-	- 50	-	-	Brunt	-	Unclear
N-Score	Miyaaki[290]	Japan	2000-05	182	Secondary care. Consecutive patients	51	40.7%	48%	Brunt	A B C	Unclear
TUE	Myers[291]	Canada	2008-09	50	Secondary care. Routine biopsy attendees.	-	-	-	Kleiner	A B C	Risk

	Study	*Country*	*Date*	*N*	*Population*	*Age, years*	*Men, %*	*DM, %*	*Histology*	*NAFLD*[1]	*Risk of bias*[2]
TUE	Myers[292]	Canada	2009-10	75	Secondary care. Routine biopsy attendees. BMI≥28kg/m^2.	-	-	-	Kleiner	-	Risk
Tetri(1-4)	Nettschwander-Tetri[293]	USA	2004-08	698	NASH CRN database and adult PIVENS trial, without cirrhosis	49	39%	31%	Kleiner	A B C D E	Unclear
AST:ALT ratio, HA, TGF-B	Palekar[294]	USA	2006[3]	80	Secondary care. Routine biopsy attendees. Retrospective review over 1 year. Military.	50.8	52.5	21.3	Brunt	A B C D E	Unclear
ARFI	Palmeri[295]	USA	2008-10	135	-	-	37.8	-	Kleiner	A F	Unclear
^{13}C-caffeine breath test	Park[296]	Australia	2009-10	48	Secondary care. Two centres. Study biopsies.	51	50.0		Kleiner	A B	None
TUE	Petta[297]	Italy	2006-10	146	Secondary care. Routine biopsy attendees.	44.1	71.2	13.7	Kleiner	A B C D E	None
BAAT	Ratziu[119]	France	1988-99	93	Secondary care. Elevated liver function tests and BMI >25kg/m^2	49.0	65.6	15.1	Metavir	A B C D	Risk
FibroTest	Ratziu[298$]	France	2004[3]	89	Secondary care. Elevated liver function tests.	-	-	-	-	-	Unclear

	Study	*Country*	*Date*	*N*	*Population*	*Age, years*	*Men, %*	*DM, %*	*Histology*	*NAFLD*[1]	*Risk of bias*[2]
FibroTest	Ratziu[241]	France	2001-04	170 97	(1) Secondary care. Routine biopsy attendees. (2) Secondary care. Elevated liver function tests. Routine biopsy.	52.8 48.5	57.6 57.2	35.9 40.0	Kleiner	A B C D	Risk
NFS	Rodriguez[299]	Spain	1999-2007	88	Secondary care. BMI ≥40 kg/m^2 undergoing bariatric surgery. >5% steatosis on biopsy.	40.6	-	-	Kleiner	A B C D E	Risk
OELF	Rosenberg[235]	UK	2004[3]	61	Secondary care. Routine biopsy attendees.	-	-	-	Scheuer Ishak	-	Risk
ELF	Rosenberg[300$]	UK	2006[3]	104	Secondary care. Routine biopsy attendees.	49	-	-	Ishak	A F	Unclear
BARD, NFS	Ruffillo[301]	Argentina	2009[3]	138	Secondary care. Single centre. Consecutive routine biopsy attendees.	49	48.6	23.2	Brunt	A B C D	None
HA, COL-IV	Sakugawa[302]	Japan	1993-2003	112	Secondary care. Multicentre. Routine biopsy attendees.	56.3	32.1	30.4	Brunt	A B C D E	Unclear
APRI, AST:ALT ratio, AST:Plt ratio, BARD, CDS, FIB4, GUCI, NFS	Shah[303]	USA	2009[3]	541	NASH CRN database and adult PIVENS trial.	-	39.9	19.4	Kleiner	A B C D	Risk
BAAT, BARD, Gholam(1), N-Score, NAFIC, NFS	Sumida[304]	Japan	2002-08	619	Secondary care. Eight centres. Routine biopsy attendees.	-	53.2	41.0	Kleiner	A B C D E	Unclear

	Study	*Country*	*Date*	*N*	*Population*	*Age, years*	*Men, %*	*DM, %*	*Histology*	*NAFLD[1]*	*Risk of bias[2]*
HA, Suzuki(1,2)	Suzuki[305]	USA	2005[3]	79	Secondary care. Single centre. Elevated liver function tests. Routine biopsy attendees.	49.0	38.0	-	Brunt	A B C D	None
MRE	Talwalker[306$]	USA	2009[3]	54	Secondary care. Routine biopsy attendees. Obese.	52	29.6	-	-	-	Risk
APRI, FM8	Tropet[307$]	France	2007[3]	397	-	49.9		-	Metavir	-	Unclear
TUE	Wong[308$]	HK	2008*	66	Secondary care. Consecutive routine biopsy attendees.	49	51.5	-	Kleiner	-	Unclear
APRI, AST:ALT ratio, HAIR, NFS	Wong[309]	HK	2004-07	162	Secondary care. Elevated liver function tests and either obesity or T2DM. Routine biopsy attendees.	46	59.3	71.0~	Brunt	A B C D E	Risk
APRI, AST:ALT ratio, BARD, FIB4, NFS, TUE	Wong[246]	HK	2003-09	246	Secondary care. Routine biopsy attendees.	51	54.9	36.2	Kleiner	A B C D E	None
APRI	Yilmaz[310]	Turkey	2011[3]	140	-	48.1	55.0	-	Kleiner	A B C D E	Unclear
HA, COL-IV	Yoneda[311]	Japan	2004-06	72	Secondary care. Single centre. Routine biopsy attendees.	52.6	-	-	Brunt	A B C D	Risk
ARFI, TUE	Yoneda[270]	Japan	2008	54	Secondary care. Single centre. Routine biopsy attendees.	50.4	46.3	-	Brunt	A B C D	Unclear

	Study	*Country*	*Date*	*N*	*Population*	*Age, years*	*Men, %*	*DM, %*	*Histology*	*NAFLD*[1]	*Risk of bias*$_2$
TUE	Yoneda[247]	Japan	2008[3]	97	Secondary care. Two centres. Routine biopsy attendees. NASH.	51.8	41.2	-	-	A B C D	Unclear
TUE	Yoneda[312#]	Japan	2007[3]	67	-	50.4			Brunt		
ELF, Younossi(1,2)	Younossi[313]	USA	2011[3]	79	Epidemiology of NAFLD (EPI-NAFLD) database	42	32.8	24.4	-	A B C D	Unclear
APRI, AST:ALT ratio, AST:plt ratio, BARD, CDS, FIB4, GUCI, NFS, P2/MS	Yu[314]	Korea	2002-09	235	Secondary care. Consecutive routine biopsy attendees.	-	53.2	13.2	Metavir	A B C D	Risk

APRI aspartate to platelet ratio index; **ALT** alanine aminotransferase; **ARFI** acoustic radiation force impulse; **AST** aspartate aminotransferase; **BMI** body mass index; **CDS** cirrhosis discriminant score; **CK18** cytokeratin-18; **COL-IV** Type IV collagen; **CRN** clinical research network; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; **GUCI** Goteburg University Cirrhosis Index; **HA** hyaluronic acid; **MRE** magnetic resonance elastography; **MS** metabolic syndrome; **NAFLD** non-alcoholic fatty liver disease; **NAS** NAFLD Activity Score; **NASH** non-alcoholic steatohepatitis; **NFS** NAFLD Fibrosis Score; **N-score** Nippon-score; **OELF** Original European Fibrosis panel; **PAF** Probability of Advanced Fibrosis; **PIVENS** Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with NASH trial; **SPEA** serum prolidase enzyme activity; **TIMP-1** metallopeptidase inhibitor 1; **TGF-β** transforming growth factor beta; **TUE** transient ultrasound elastography (Fibroscan); **T2DM** type 2 diabetes mellitus; **USS** ultrasound scan.

[1] NAFLD defined as: A – alcohol statement; B- viral hepatitis statement; C – autoimmune statement; D – metabolic statement; E – hepatosteatotic drug statement; F – general statement of exclusion of other liver diseases; [2] QUADAS II; [3]date of publication; [$]Abstract only, no full paper identifiable; [#]Letter, no full paper identifiable; [e]Estimation cohort; [v]Validation cohort

Table 2-5 Components of panel markers of liver fibrosis

Aspartate to platelet ratio index
[(AST (U/L) / upper limit of normal (U/L)) / platelet ($x10^9$/L)]*100
AST:platelet ratio
Sum of:
Age score: <30 = 0; 30–39 = 1; 40–49 = 2; 50–59 = 3;60–69 = 4; ≥70 = 5
Platelet count score: ≥225 ($x10^9$) = 0; 200–224 ($x10^9$) = 1; 175–199 ($x10^9$) = 2; 150–174 ($x10^9$) = 3; 125–149 ($x10^9$) = 4; >125 ($x10^9$) =5
(Possible value 0–10)
AST:ALT ratio
AST (U/L) / ALT (U/L)
BAAT
Sum of: BMI ≥28 score 1, age ≥50 score 1, ALT ≥2x upper normal limit score 1, and triglycerides ≥1.7mmol/l score 1, with total score being 0-4.
BARD
Sum of: BMI ≥28 score 1, ALT/AST ratio ≥0.8 score 2, and diagnosis of diabetes score 1
Cirrhosis discriminant score
Sum of:
Platelet count score:: ≥340 ($x10^9$/L) =0; 280–339 ($x10^9$/L) =1; 220–279 ($x10^9$/L) =2; 160–219 ($x10^9$/L) =3; 100–159 ($x10^9$/L) =4; 40–99 ($x10^9$/L) =5; <40 ($x10^9$/L) =6
AST/ALT ratio score: 1.7 = 0; 1.2–1.7 = 1; 0.6–1.19 = 2; <0.6 = 3
INR score: <1.1 = 0; 1.1–1.4 = 1; >1.4 = 2
(Possible value 0–11).
dosSantos-1
Laminin + HA

dosSantos-2
Laminin + type IV colagen
dosSantos-3
Laminin + AST
Enhanced Liver Fibrosis panel
-7.412 + (ln(HA)*0.681) + (ln(P3NP)*0.775) + (ln(TIMP1)*0.494)
Enhanced Liver Fibrosis panel + modified NAFLD Fibrosis Score
-2.722 + 1.482*ELF + 0.062*BMI (kg/m2) + 1.241*diabetes/IFG (yes=1, no=0) - 0.590*AST/ALT ratio - 0.002*platelets ($x10^9$/L) - 0.043*alb (g/L)
FibroMeter NAFLD
11.623 + 0.418*glucose (mmol/l) + 0.070*AST (UI/l) + 0.001*ferritin (lg/l) - 0.010*platelet(G/l) - 0.026*ALT (UI/l) + 0.046*weight (kg) + 0.084*age (yr)
FIBROspect II
HA, TIMP-1 and alpha-2 macroglobulin (formula not available)
FibroTest
4.467*$\log_{10}$alpha2macroglobulin(g/L)-1.357*$\log_{10}$haptoglobin(g/L)+1.017*$\log_{10}$GGT(U/L)+0.0281*age(years)+1.737*$\log_{10}$bilirubin(micromole/L)-1.184*apolipoproteinA1(g/L)+0.301*sex(female=0, male=1)-5.54
Fibrosis-4 Index
[age(years)*AST(U/L)]/[platelets($x10^9$/L)*$\sqrt{}$ALT(U/L)^2)
FM8
Cholesterol, triglyceride, AST, ALT, GGT, gammaglobulins, age and weight (formula not published)
Gholam
5 + 2.45*ln ALT (U/L) - 38.55* (1/HbA1C)
Goteburg University Cirrhosis Index
[AST(U/L)/upper limit of normal]*INR*platelet($x10^9$/L)*100

HAIR
Sum of: hypertension (BP ≥140/90 or on antihypertensive meds)=1, ALT>40 U/L=1, and insulin resistance (IR index>5)=1
Hepascore
Exp[-4.185818-0.0249*age(years)+0.7464*sex(female=, male=)+1.0039*alpha2macroglobulin+0.0302*HA+0.0691*bilirubin-0.0012*GGT]
Manousou(1)
Ferritin ≥240 ng/ml and BMI ≥28.2kg/m2 = positive score
Manousou(2)
Ferritin ≥240 ng/ml and BMI ≥28.2kg/m2 and type 2 diabetes = positive score
NAFIC
Sum of: Ferritin ≥200 ng/ml (female) or ≥300 ng/ml (male)=1, immunoreative insulin ≥10 microU/ml=1 and COL-IV ≥5,0 ng/ml=2
NAFLD Fibrosis Score
-1.675 + 0.037*age (years) + 0.094*BMI (kg/m2) + 1.13*IFG/diabetes (yes=1, no=0) + 0.99*AST/ALT ratio - 0.013*platelet (x10^9/l) - 0.66*albumin (g/dl)
Modified NFS:
6.375 + 0.062*BMI (kg/m2) + 1.745*diabetes/IFG (yes=1, no=0) - 1.103*AST/ALT ratio - 0.037*age (years) - 0.005*platelets (x10^9/l) - 0.093*albumin (g/L)
Nippon-Score
Sum of:
Female=1, BMI≥25kg/m^2=1, type 2 diabetes=1, systolic blood pressure>130mmHg or diastolic blood pressure>85mmHg=1 (total score range 0-4)
Original European Liver Fibrosis panel
-6.38 – (ln(age)*0.14) + (ln(HA)*0.616) + (ln(P3NP)*0.586) + (ln(TIMP1)*0.472)
P2/MS
Platelet count(x10^9/L)/[monocyte fraction(%)*segmented neutrophil fraction(%)]

Probability of Advanced Fibrosis
Sex, diabetes, ALT, AST (formula not published)
Susuki-1
Age, obesity, AST:ALT ratio >1 and diabetes (formula not published)
Susuki-2
Age, obesity, AST:ALT ratio >1, diabetes and HA (formula not published)
Tetri-1
AST, ALT, AST:ALT ratio (formula not published)
Tetri-2
AST, ALT, AST:ALT ratio, age, race, gender, ethnicity (formula not published)
Tetri-3
AST, ALT, AST:ALT ratio, age, race, gender, ethnicity, hypertension, Type 2 diabetes, body mass index, waist circumference, waist/hip ratio, acanthosis nigricans (formula not published)
Tetri-4
AST, ALT, AST:ALT ratio, age, race, gender, ethnicity, hypertension, Type 2 diabetes, body mass index, waist circumference, waist/hip ratio, acanthosis nigricans, ALP, GGT, globulin, albumin, total and direct bilirubin, international normalized ratio, hematocrit, white blood cells, platelet count, total cholesterol, triglyercides, HDL cholesterol, LDL cholesterol, HbA1c, HOMA-IR, fasting serum glucose, fasting serum insulin, autoimmune markers (ANA, AMA, ASMA), metabolic syndrome, ferritin (formula not published)
Younossi-1
Type 2 diabetes, sex, BMI, triglycerides, CK18 (formula not published)
Younossi-2
Type 2 diabetes, AST, triglycerides, TIMP1 (formula not published)

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **BMI** body mass index; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis panel; **GGT** gammaglutamyl transferase; **HA** hyaluronic acid; **INR** international normalised ratio; **P3NP** procollagen type III N-terminal peptide; **TIMP1** tissue inhibitor of metalloproteinase-1.

2.4.2 Methodological quality

The overall rating using the Quality Assessment of Diagnostic Accuracy Studies II approach found 8 studies to have no risk of bias, 23 studies to have a definite risk of bias and 26 studies where the risk of bias was unclear. Full details are included in Appendix G.

For the studies with a definite risk of bias 16/23 were due to concerns over the patients group selected (e.g. gastric bypass participants, NASH patients, specific ethnic group) and 9/23 were due to a time interval >3 months between the index and reference test (2 studies had both risks).

For studies where the risk of bias was unclear 15/26 were because of incomplete description of the study population and 22/26 were due to failure to disclose the interval between the index and reference test. Again a number of studies had both risks.

2.4.3 Findings

Any fibrosis

Table 2-6 summarises the characteristics of included studies of any fibrosis.

The prevalence of underlying fibrosis ranged from 26 to 81%.

For any fibrosis (F0 vs F1-4) there were 25 different markers trialled in 38 assessments. For mild fibrosis 18/25 (72%) markers were only considered once. The highest numbers of different studies was for TUE (5), HA (4) and ELF (3). In general, when a marker was assessed in more than one different study the resulting AUROC statistics were similar with the exception of HA and TUE which are discussed below.

For TUE the five assessments totalled 414 participants. The AUROC statistics ranged from 0.88-0.93 in four studies and a fifth study found a lower score of 0.78. All five studies optimised similar cut-offs (5.3-5.9 kPa) which in a low fibrosis (5%)

prevalence cohort gave a low PPV of 9-42%, and an excellent NPV of 98-100%. When the fibrosis prevalence was increased (30%) the PPV improved to 45-76% and NPV fell to 89-96%.

The four HA studies contained in total 254 participants with wide ranging AUROC varying from 0.67-0.98. Only three reported optimal cut-off values and these varied from 25 -149 ng/ml. In taking the higher cut-off Lydatakis[286] was able to produce a sensitivity and specificity both of 96%, however the lower cut-off from dos Santos[274] had values of 82% and 68% respectively.

The three ELF studies total 375 participants with consistent AUROC of 0.76-0.82. Guha and colleagues[236] improved this to 0.84 by combining ELF with the NFS. Again reported cut-offs and their corresponding sensitivity and specificity were varied. Despite different cut-offs (-0.21 vs 0.18) Rosenberg[300] and Guha[236] found similar sensitivity and specificity of 61-69% and 80-82% respectively.

Of the remaining markers the best AUROC statistics were for complex formulas derived by dos Santos[274] and Gholam[277] (all >0.80) and the lowest were for individual markers (TIMP-1 0.57 and YKL-40 0.62).

Overall there was a clear improvement in AUROC for panel markers and TUE over individual markers. In a low fibrosis prevalence (5%) hypothetical cohort the PPV values were very poor for all markers (often <20%). This improved with a higher prevalence of fibrosis to between 40-70%. The NPV was very good for all markers.

Table 2-6 Non-invasive markers of ANY (F0 vs F1-4) liver fibrosis

	Study	*AU ROC (95%CI)*	*Fibrosis prevalence*	*Cut off*	*Sensitivity*	*Specificity*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
Individual markers										
ALT	Kayadibi[281]	0.76	26.4%	26 U/L	68	60	10	97	42	81
AST	Dos Santos[274]	0.73	36.7%	44 U/L	64	79	13	97	56	83
AST	Kayadibi[281]	0.73	26.4%	25 U/L	67	68	9	97	48	83
CK18	Malik[86]	0.67 (0.63,0.71)	69.5%	-	-	-	-	-	-	-
HA	Lydatakis[286]	0.98 (0.93,1.01)	46.0%	149 ng/mL	96	96	56	100	91	99
HA	Malik[86]	0.73 (0.68,0.78)	69.5%	45 ng/ml	-	-	-	-	-	-
HA	Dos Santos[274]	0.73	36.7%	24.6 ng/ml	82	68	12	98	53	91
HA	Suzuki[305]	0.67 (0.55,0.80)	74.7%	-	-	-	-	-	-	-
Laminin	Dos Santos[274]	0.87	36.7%	282 ng/mL	82	89	29	99	76	95
Laminin	Lydatakis[286]	0.79 (0.66,0.92)	46.0%	293 ng/ml	74	74	14	99	55	87
SPEA	Kayadibi[281]	0.84 (0.73,0.94)	26.4%	1134	84	82	19	99	66	92
TIMP-1	Malik[86]	0.59 (0.53,0.65)	69.5%	-	-	-	-	-	-	-
COL-IV	Dos santos[274]	0.80	36.7%	145 ng/mL	64	89	69	98	70	85
YKL-40	Malik[86]	0.62 (0.55,0.69)	69.5%	-	-	-	-	-	-	-
Panel markers										
APRI	Younossi[313]	0.70 (0.58,0.80)	49.4%	0.124	92	29	7	100	36	91
				0.5	6	97	0	95	50	71
APRI	Yilmaz[310]	0.63 (0.53,0.72)	-	0.45	60	73	10	97	50	82

	Study	*AU ROC (95%CI)*	*Fibrosis prevalence*	*Cut off*	*Sensitivity*	*Specificity*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
AST:ALT ratio	Kayadibi[281]	0.67	26.4%	1.09	63	60	7	97	40	79
dosSantos-1	Dos santos[274]	0.87	36.7%	-	73	95	44	99	88	89
dosSantos-2	Dos santos[274]	0.87	36.7%	-	64	100	100	98	100	86
dosSantos-3	Dos santos[274]	0.80	36.7%	-	55	95	38	98	85	84
ELF	Rosenberg[300]	0.82 (0.74,0.90)	51.9%	0.18	69	82	15	98	62	86
ELF	Younossi[313]	0.76 (0.65,0.85)	49.4%	-0.675	72	72	13	99	52	86
				0.573	28	85	7	95	44	73
ELF	Guha[236]	0.76 (0.69,0.83)	58.9%	-0.207	61	80	14	97	56	82
ELF+NFS*	Guha[236]	0.84 (0.76,0.92)	58.9%	-5.002	92	52	10	100	45	95
				-3.346	60	91	25	98	75	84
Gholam-1	Gholam[277]	0.90		6.60	83	82	19	99	66	92
Tetri-1	Tetri[293]	0.72 (0.67,0.76)	73.1%	-	-	-	-	-	-	-
Tetri-2	Tetri[293]	0.74 (0.70,0.78)	73.1%	-	-	-	-	-	-	-
Tetri-3	Tetri[293]	0.78 (0.74,0.82)	73.1%	-	-	-	-	-	-	-
Tetri-4	Tetri[293]	0.84 (0.80,0.87)	73.1%	-	-	-	-	-	-	-
NFS*	Guha[236]	0.79 (0.69,0.88)	58.9%	-0.707	92	50	10	100	44	95
				1.140	32	91	18	97	63	76
NFS	Younossi[313]	0.71 (0.59,0.81)	49.4%	−1.633	100	0	5	100	30	100
				-0.166	85	34	6	97	36	86
OELF	Younossi[313]	0.76 (0.65,0.85)	49.4%	-0.488	92	38	9	100	39	93
				1.213	28	90	10	96	53	74

	Study	*AU ROC (95%CI)*	*Fibrosis prevalence*	*Cut off*	*Sensitivity*	*Specificity*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
Younossi-1	Younossi[313]	0.80 (0.68,0.88)	49.4%	0.219	91	44	9	100	41	91
				0.424	61	72	10	97	47	81
Imaging										
TUE	Yoneda[247]	0.93	81.4%	5.9 kPa	86	89	29	99	76	94
TUE	Yoneda[312]	0.88	77.6%	5.6 kPa	83	81	18	99	66	92
TUE	Lupsor[285]	0.88 (0.78,0.95)	65.3%	5.3 kPa	93	72	42	100	58	96
TUE	Miette[289]	0.88[B]	67.4%	5.6 kPa	82	81	18	99	66	92
TUE	Gaia[276]	0.78 (0.67,0.88)	68.1%	5.5 kPa	84	57	9	98	45	89

ALT alanine aminotransferase; **APRI** aspartate to platelet ratio index; **AST** aspartate aminotransferase; **AUROC** area under the receiver operator curve; **CK18** cytokeratin-18; **COL-IV** Type IV collagen; **ELF** Enhanced Liver Fibrosis panel; **HA** hyaluronic acid; **NAFLD** non-alcoholic fatty liver disease; **NFS** NAFLD Fibrosis Score; **NPV** negative predictive value; **OELF** Original ELF panel; **PPV** positive predictive value; **SPEA** serum prolidase enzyme activity; **TIMP-1** metallopeptidase inhibitor 1; **TUE** transient ultrasound elastography.

Moderate fibrosis

Table 2-7 summarises the characteristics of included studies of moderate fibrosis.

The prevalence of underlying fibrosis ranged from 9 to 53%.

For moderate fibrosis (F1 vs F2-4) there were 34 different markers trialled in 59 assessments. For moderate fibrosis 16/34 (47%) markers were only considered once. The highest numbers of different studies was for NFS (17) and TUE (15). Unlike for any fibrosis, when a marker was assessed n more than one different study the resulting AUROC statistics were in poor agreement with the exception of TUE. For example, from seven assessments of APRI the AUROC spread from 0.56-0.87.

The 17 assessments of the NFS totalled 1275 participants. The AUROC ranged from 0.79-0.94. The underlying fibrosis prevalence of the investigated cohorts ranged from 25-53%. The cut-offs suggested varied but in general corresponded to a sensitivity of 70-90% and a specificity of 60-80%. Seven studies had a cut-off between 6.5-7.5 kPa corresponding to sensitivities 67-89% and specificities 70-91%. In the low fibrosis prevalence group the PPV was <31% (except one assessment) and the specificities >96%. For the higher fibrosis prevalence group the PPV rose to above 50% with an NPV >80%.

Other notable high AUROCs were for FibroMeter NAFLD (0.94)[272], MRE (0.90)[306], FM8 (0.87)[307], HA (0.87)[315], investigator derived formulas from Manousou (>0.84)[287], Elf (>0.81)[236,300]. The only assessment of the C-caffeine breath test was for moderate fibrosis with an AUROC of 0.74.

Notable poor AUROC occurred for AST (0.60)[278] and HAIR (<0.60)[304,309].

Table 2-7 Non-invasive markers of MODERATE (F0-1 vs F2-4) liver fibrosis

	Study	*AU ROC*	*Fibrosis prevalence*	*Cut off*	*Sensitivity*	*Specificity*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
Individual markers										
AST	Guajardo-Salinas[278]	0.60 (0.37,0.83)	8.7%	-	-	-	-	-	-	-
HA	Suzuki[305]	0.87 (0.80,0.95)	32.9%	-	-	-	-	-	-	-
Panel markers										
APRI	Cales[272]	0.87	27.6%	-	66	91	25	97	77	86
APRI	Tropet[307]	0.79	32.5%	-	-	-	-	-	-	-
APRI	Adams[271]	0.73 (0.64,0.82)	42.0%	-	72	70	13	99	51	86
APRI	Kelleher[282]	0.73 (0.61,0.84)	49.6%	-	-	-	-	-	-	-
APRI	Shah[303]	0.70 (0.67,0.74)	44.7%	-	-	-	-	-	-	-
APRI	Wong[309]	0.62 (0.51,0.72)	25.3%	-	-	-	-	-	-	-
APRI	Loaeza[229]	0.56	-	-	-	-	-	-	-	-
AST:ALT ratio	Kelleher[282]	0.67 (0.58,0.77)	49.6%	-	-	-	-	-	-	-
AST:ALT ratio	Wong[309]	0.63 (0.53,0.73)	25.3%	-	-	-	-	-	-	-
BAAT	Ratziu[119]	0.84	30.1%	1	100	11	6	100	33	100
				2	100	46	9	100	44	100
				3	71	80	17	99	60	86
				4	14	100	100	96	100	73
BAAT	Sumida[304]	0.59	24.6%	-	-	-	-	-	-	-
BARD	Sumida[304]	0.69	24.6%	-	-	-	-	-	-	-
BARD	Shah[303]	0.68 (0.62,0.72)	44.7%	-	-	-	-	-	-	-

	Study	*AU ROC*	*Fibrosis prevalence*	*Cut off*	*Sensitivity*	*Specificity*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
CDS	Shah[303]	0.63 (0.59,0.67)	44.7%	-	-	-	-	-	-	-
ELF	Rosenberg[300]	0.91 (0.85,0.969)	31.7%	0.24	88	79	17	91	63	93
ELF	Guha[236]	0.82 (0.75,0.88)	40.1%	-0.107	70	80	17	99	60	86
ELF+NFS*	Guha[236]	0.93 (0.88,0.99)	40.1%	-0.995	89	86	24	99	73	95
				-0.016	79	91	31	99	80	91
FibroMeter	Cales[272]	0.94	27.6%	-	79	96	50	99	88	91
FIBROspect II	Guajardo-Salinas[278]	0.78 (0.62,0.94)	8.7%	20	100	42	100	96	100	74
FibroTest	Ratziu[241]	0.86 (0.77,0.91)	23.5%	0.30	83	78	16	99	63	92
				0.70	18	98	33	96	63	73
FibroTest	Lassailly[284]	0.82 (0.67,0.90)	6.9%	0.48	5	100	-	95	100	71
FibroTest	Ratziu[241]	0.75 (0.61,0.83)	32.0%	0.30	71	74	15	99	54	85
				0.70	13	98	33	96	80	73
FibroTest	Adams[271]	0.72 (0.62,0.81)	42.0%	-	73	60				
FIB4	Shah[303]	0.75	44.7%	-	80	56	9	98	44	87
FM8	Tropet[307]	0.87	-	-	-	-	-	-	-	-
Gholam-1	Sumida[304]	0.79	24.6%	-	-	-	-	-	-	-
GUCI	Shah[303]	0.71 (0.63,0.74)	44.7%	-	-	-	-	-	-	-
HAIR	Sumida[304]	0.59	-	-	-	-	-	-	-	-
HAIR	Wong[309]	0.51 (0.41,0.61)	25.3%	-	-	-	-	-	-	-
Hepascore	Adams[271]	0.75 (0.66,0.84)	42.0%	-	56	94	33	98	81	84
Manousou-1	Manousou[287]	0.85 (0.78,0.93)	24.2%	+ve	86	77	15	99	62	93

	Study	*AU ROC*	*Fibrosis prevalence*	*Cut off*	*Sensitivity*	*Specificity*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
Manousou-1	Manousou[287]	0.87 (0.80,0.93)	32.4%	+ve	82	79	17	99	63	92
Manousou-2	Manousou[287]	0.85 (0.78,0.93)	24.2%	+ve	90	72	14	99	57	94
N-Score	Sumida[304]	0.72	24.6%	-	-	-	-	-	-	-
NAFIC	Sumida[304]	0.83	24.6%	3	84	74	14	99	58	91
NFS	Cales[272]	0.88	27.6%	-	61	96	43	98	86	85
NFS*	Guha[236]	0.86 (0.78,0.94)	40.1%	-1.633	89	57	9	98	47	93
				-0.166	68	89	23	98	71	86
NFS	Sumida[304]	0.82	24.6%	-1.455	86	69	12	99	54	92
				0.676	23	96	20	96	70	74
NFS	Shah[303]	0.69 (0.65,0.73)	44.7%	-	-	-	-	-	-	-
NFS	Wong[309]	0.67 (0.57,0.76)	25.3%	-1.455	37	84	12	96	50	76
				0.676	0	98	0	95	0	70
Imaging										
MRE	Talwalkar[306]	0.90	48.1%	4.2 kPa	78	94	40	99	85	90
TUE	De Ledinghen[273]	0.94 (0.91,0.97)	38.0%	7.0 kPa	77	77	54	99	59	89
TUE	Yoneda[312]	0.88	49.3%	6.65 kPa	82	91	31	99	81	93
TUE	Yoneda[247]	0.87	52.6%	6.65 kPa	88	74	14	99	59	93
TUE	Miette[289]	0.87[B]	-	6.65 kPa	81	91	31	99	80	91
TUE	Kelleher[282]	0.86 (0.79,0.92)	49.6%	10.0 kPa	88	72	13	99	57	93
TUE	Myers[292]	0.86 (0.77,0.94)[M]	-	7.8 kPa	84	79	17	99	63	92
TUE	Myers[292]	0.85 (0.76,0.94)[XL]	-	6.4 kPa	81	66	11	98	50	88

	Study	*AU ROC*	*Fibrosis prevalence*	*Cut off*	*Sensitivity*	*Specificity*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
TUE	Myers[291]	0.84 (0.73,0.95)	34.0%	7.7 kPa	94	61	12	100	51	96
TUE	Wong[246]	0.84 (0.79,0.90)		5.8 kPa	91	50	10	100	44	92
			41.1%	7.0 kPa	79	76	15	99	59	90
				9.0 kPa	53	92	27	98	73	82
TUE	Miette[289]	0.84[A]	50.0%	5 kPa	96	44	9	100	43	97
TUE	Freidrich-Rust[275]	0.82 (0.70,0.95)[XL]	-	-	-	-	-	-	-	-
TUE	Gaia[276]	0.80 (0.70,0.91)	45.8%	7.0 kPa	76	80	17	99	62	89
TUE	Freidrich-Rust[275]	0.80 (0.64,0.96)[M]	-	-	-	-	-	-	-	-
TUE	Lupsor[285]	0.79 (0.67,0.88)	25.0%	6.8 kPa	67	84	17	98	65	86
TUE	Petta[297]	0.79	46.6%	7.25 kPa	69	70	10	97	50	84
‘Other’ markers										
^{13}C-caffiene breath test	Park[296]	0.74 (0.60,0.88)	47.9%	-	-	-	-	-	-	-

APRI aspartate to platelet ratio index; **ALT** alanine aminotransferase; **AST** aspartate aminotransferase; **BMI** body mass index; **CDS** cirrhosis discriminant score; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; **GUCI** Goteburg University Cirrhosis Index; **HA** hyaluronic acid; **MRE** magnetic resonance elastography; **NAFLD** non-alcoholic fatty liver disease; **NFS** NAFLD Fibrosis Score; **NPV** negative predictive value; **N-score** Nippon-score; **OELF** Original European Fibrosis panel; **PPV** positive predictive value; **TUE** transient ultrasound elastography.

Significant fibrosis

Table 2-8 summarises the results of included studies of significant fibrosis.

The prevalence of underlying fibrosis ranged from 3 to 43%.

For significant fibrosis (F0-2 vs F3-4) there were 37 different markers trialled in 118 assessments. For moderate fibrosis 16/34 (47%) markers were only considered once. The most frequently assessed markers were: NFS (17), TUE (16), APRI (10), AST:ALT ratio (9), and BARD (9).

Again, when a marker was assessed i n more than one different study the resulting AUROC statistics were widely varied. For example, 17 assessments of NFS (including 4480 participants) produced AUROC spread from 0.59-0.96.

Five assessment of HA had similar cut-offs (33-50 ng/ml) with corresponding sensitivities 69-100% and specificities 66-89%. For a low fibrosis prevalence cohort this gave a PPV general of <20% and an NPV >97%. By increasing the fibrosis prevalence the PPV rose to <66%.

Overall the AUROC for the AST:ALT ratio was average to good (0.59-0.83). Three assessments used a cut-off of 1.0 with sensitivities 21-52% and specificities 78-90%. Four assessments used a cut-off of 0.8 with marginally improved sensitivities of 40-74% and specificities of 62-90%.

Four assessments of BARD reported for a cut-off of 2 (AUROC 0.65-0.81). The sensitivities were 51-89% and specificities 34-77%.

FIB4 was assessed five times (AUROC 0.75-0.96). as cut-offs increased from 1.3 to 2.67 and 3.25 the sensitivities fell (65-85%, 21-33% and 26-71% respectively) and specificities improved (65-80%, 96-80% and 96-80% respectively).

FibroTest had consistently very good AUROC from four studies (0.81-94). For a cut-off of 0.30 (three assessments) the sensitivities were 88-100% and specificities

69-73%. For a higher cut-off of 0.70 (two studies) the sensitivity was 25% and specificity 97-99%.

From the 17 NFS studies there were seven reporting a low cut-off of -1.455. These had a sensitivity of 39-100% and a specificity of 31-99%. Nine studies used a higher cut-off of 0.676 with sensitivity 0-62% (majority <50%) and specificity 31-99% (mainly >90%).

For TUE nine assessments used a lower cut-off of 7.9-8.7 kPa with sensitivity 46-91% and specificity 75-92%. Four assessments used a cut-off of 9.8-10.4 kPa with similar results: sensitivity 46-91% and specificity 75-92%.

Markers with notable high AUROC were ARFI (0.90-0.97), P2/MS (0.94) and FM8 (0.94). The markers with consistently low AUROC were HAIR (0.50-0.57) and BAAT (0.53-0.57).

Overall

For all levels of fibrosis across all of the cut-offs assessed and all of the varying sensitivity and specificity there was consistently poor PPV and excellent NPV, meaning that many people with a positive test result would be falsely diagnosed with liver fibrosis but that a negative test would be highly reassuring for the absence of fibrosis. The PPV improved with increased fibrosis prevalence. As the fibrosis stage being determined increased the diagnostic test accuracy increased.

Table 2-8 Non-invasive markers of SIGNIFICANT (F0-2 vs F3-4) liver fibrosis

	Study	*AU ROC (95%CI)*	*Prevalence fibrosis*	*Cut off*	*Sensitivity %*	*Specificity %*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
Single markers										
CK18	Malik[86]	0.71 (0.68,0.74)	26.3%	-	-	-	-	-	-	-
HA	Kaneda[280]	0.97	27.0%	42 ng/mL	100	89	33	100	79	100
HA	Suzuki[305]	0.89 (0.81,0.97)	25.3%	46.1 ng/mL	85	80	17	99	65	93
HA	Palekar[294]	0.89 (0.76,1.00)	17.5%	45.3 micrg/L	86	80	17	99	65	93
HA	Sakugawa[302]	0.80 (0.65,0.85)	42.9%	50 ng/mL	69	83	16	98	64	87
HA	Malik[86]	0.77 (0.73,0.81)	26.3%	-	-	-	-	-	-	-
HA	Yoneda[311]	0.75	25.0%	32.5 ng/dL	78	66	11	98	49	87
TGR-B	Palekar[294]	0.67 (0.50,0.84)	17.5%	-	-	-	-	-	-	-
TIMP-1	Malik[86]	0.60 (0.56,0.64)	26.3%	-	-	-	-	-	-	-
COL-IV	Kaneda[280]	0.87	27.0%	-	-	-	-	-	-	-
COL-IV	Sakugawa[302]	0.82 (0.74,0.90)	42.9%	5.0 ng/mL	81	71	13	99	55	89
COL-IV	Yoneda[311]	0.77	25.0%	4.3 ng/dL	89	60	10	98	49	93
YKL-40	Malik[86]	0.64 (0.59,0.69)	26.3%	-	-	-	-	-	-	-
Panel markers										
APRI	Cales[272]	0.86	18.7%	-	-	-	-	-	-	-
APRI	Kruger[283]	0.85	17.1%	0.98	75	86	24	99	70	90
APRI	Tropet[307]	0.83	-	-	-	-	-	-	-	-
APRI	Yu[314]	0.83 (0.74,0.91)	3.0%	-	-	-				
APRI	Adams[271]	0.78 (0.68,0.88)	24.4%	0.42	72	70	13	99	51	86

	Study	AU ROC (95%CI)	Prevalence fibrosis	Cut off	Sensitivity %	Specificity %	PPV 5%	NPV fibrosis	PPV 30%	NPV fibrosis
APRI	Wong[246]	0.74 (0.67,0.82)	22.8%	0.5	65	72	10	97	50	83
				1.5	6	97	0	95	50	71
APRI	Shah[303]	0.73	23.1%	-	-	-	-	-	-	-
APRI	Wong[309]	0.70 (0.55,0.84)	11.1%	-	-	-	-	-	-	-
APRI	Younossi[313]	0.68 (0.56,0.79)	20.3%	0.121	93	20	6	100	33	88
				0.5	7	97	0	95	50	71
APRI	McPherson[228]	0.67 (0.54,0.80)	18.6%	1	27	89	9	96	50	74
APRI	Loaeza[229]	0.57	-	-	-	-	-	-	-	-
AST:ALT ratio	McPherson[228]	0.83	18.6%	0.8	74	78	16	99	59	87
				1	52	90	25	98	70	82
AST:ALT ratio	Yu[314]	0.79 (0.69,0.89)	3.0%	-	-	-	-	-	-	-
AST:ALT ratio	Palekar[294]	0.74 (0.61,0.88)	17.5%	0.79	71	70	13	99	50	84
AST:ALT ratio	Shah[303]	0.74 (0.69,0.79)	23.1%	-	-	-	-	-	-	-
AST:ALT ratio	Miao[288]	0.66	3.1%	-	-	-	-	-	-	-
AST:ALT ratio	Wong[246]	0.66 (0.58,0.74)	22.8%	0.8	40	80	10	96	46	76
				1.0	21	90	10	96	46	72
AST:ALT ratio	Kruger[283]	0.61	17.1%	0.8	58	62	8	97	39	77
AST:ALT ratio	Kallwitz[279]	0.59	-	1	41	78	9	96	40	74
AST:ALT ratio	Wong[309]	0.58 (0.44,0.71)	11.1%	-	-	-	-	-	-	-
AST:Plt ratio	Yu[314]	0.83 (0.74,0.91)	3.0%	-	-	-	-	-	-	-
AST:Plt ratio	Shah[303]	0.72 (0.67,0.77)	23.1%	-	-	-	-	-	-	-

	Study	AU ROC (95%CI)	Prevalence fibrosis	Cut off	Sensitivity %	Specificity %	PPV 5%	NPV fibrosis	PPV 30%	NPV fibrosis
BAAT	Kaneda[280]	0.57	27.0%	-	-	-	-	-	-	-
BAAT	Sumida[304]	0.53	10.8%	-	-	-	-	-	-	-
BARD	Harrison[233]	0.81[#]	22.0%	2	-	-	-	-	-	-
BARD	McPherson[228]	0.77	18.6%	2	89	44	7	98	41	91
BARD	Fujii[234]	0.73	37.7%	-	-	-	-	-	-	-
BARD	Sumida[304]	0.73	10.8%	-	-	-	-	-	-	-
BARD	Shah[303]	0.70 (0.64,0.75)	23.1%	-	-	-	-	-	-	-
BARD	Wong[246]	0.69 (0.61,0.77)	22.8%	2	62	66	9	97	43	79
BARD	Yu[314]	0.69 (0.54,0.84)	3.0%	-	-	-	-	-	-	-
BARD	Ruffillo[301]	0.67	26.8%	2	51	77	12	97	48	78
BARD	Kallwitz[279]	0.65	-	2	82	34	6	97	35	83
CDS	Yu[314]	0.94 (0.86,1.00)	3.0%	-	-	-	-	-	-	-
CDS	Shah[303]	0.67 (0.61,0.72)	23.1%	-	-	-	-	-	-	-
ELF	Rosenberg[300]	0.93 (0.87,0.98)	24.0%	0.62	83	90	31	99	78	93
ELF	Guha[236]	0.90 (0.84,0.96)	22.9%	0.358	80	90	31	99	70	91
ELF	Younossi[313]	0.65 (0.53,0.75)	20.3%	0.358	44	73	7	96	41	75
ELF+NFS	Guha[236]	0.98 (0.96,1.00)	22.9%	-0.283	91	96	56	100	90	96
				0.003	86	99	80	99	96	95
FIB4	Yu[314]	0.96 (0.94,0.99)	3.0%	1.45	100	74	17	100	63	100
				3.25	71	96	50	99	88	88

	Study	*AU ROC (95%CI)*	*Prevalence fibrosis*	*Cut off*	*Sensitivity %*	*Specificity %*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
FIB4	McPherson[228]	0.86	18.6%	1.30	85	65	11	98	52	92
				3.25	26	98	33	96	89	76
FIB4	Wong[246]	0.80 (0.74,0.87)	22.8%	1.3	65	80	14	97	59	85
				2.67	21	96	20	96	67	74
FIB4	Shah[303]	0.80 (0.76,0.85)	23.1%	1.3	74	71	13	99	52	86
				2.67	33	98	50	97	91	78
FIB4	Miao	0.75	3.1%	-	-	-	-	-	-	-
FibroMeter	Cales[272]	0.94 (0.91,0.98)	18.7%	-	-	-	-	-	-	-
FibroTest	Ratziu[298]	0.94 (0.89,0.99)	11.2%	0.30	100	73	16	100	61	100
				0.60	60	97	50	98	90	85
FibroTest	Ratziu[241]	0.92 (0.83,0.96)	11.8%	0.30	95	71	15	100	59	98
				0.70	25	97	25	96	80	76
FibroTest	Adams[271]	0.82 (0.73,0.92)	24.4%	-	-	-	-	-	-	-
FibroTest	Ratziu[241]	0.81 (0.64,0.91)	16.5%	0.30	88	69	12	99	54	92
				0.70	25	99	50	96	89	76
FM8	Tropet[307]	0.94	32.5%	-	-	-	-	-	-	-
Gholam-1	Sumida[304]	0.73	10.8%	-	-	-	-	-	-	-
GUCI	Yu[314]	0.86 (0.78,0.94)	3.0%	-	-	-	-	-	-	-
GUCI	Shah[303]	0.74 (0.70,0.79)	23.1%	-	-	-	-	-	-	-
HAIR	Sumida[304]	0.57	-	-	-	-	-	-	-	-

	Study	AU ROC (95%CI)	Prevalence fibrosis	Cut off	Sensitivity %	Specificity %	PPV 5%	NPV fibrosis	PPV 30%	NPV fibrosis
HAIR	Wong[309]	0.50 (0.36,0.65)	11.1%	-	-	-	-	-	-	-
Hepascore	Adams[271]	0.82 (0.72,0.93)	24.4%	0.50	56	94	33	98	81	84
NAFIC	Sumida[304]	0.87	10.8%	3	84	82	19	99	66	92
NAS	Malik[86]	0.79 (0.75,0.83)	26.3%	-	-	-	-	-	-	-
NFS	Yu[314]	0.96 (0.94,0.99)	3.0%	-1.455	100	75	17	100	64	100
				0.676	14	99	50	96	80	73
NFS	Cales[272]	0.93	18.7%	-	-	-	-	-	-	-
NFS	Guha[236]	0.89 (0.81,0.97)	22.9%	-2.382	91	59	11	100	48	93
				-0.833	77	93	36	99	82	90
NFS	Angulo[243]	0.88 (0.85,0.92)	26.0%[e]	0.68	51	98	56	97	94	82
NFS	Sumida[304]	0.85	10.8%	-1.455	92	62	11	99	51	96
				0.676	33	95	29	97	77	77
NFS	Fujii[234]	0.84	37.7%	-	-	-	-	-	-	-
NFS	Angulo[243]	0.82 (0.76,0.88)	28.9%[v]	0.68	43	96	33	97	81	80
NFS	McPherson[316]	0.81	18.6%	-1.455	78	58	9	98	44	85
				0.676	33	98	50	97	91	78
NFS	Miao[288]	0.79	3.1%	-	-	-	-	-	-	-
NFS	Shah[303]	0.77 (0.72,0.82)	23.1%	-	-	-	-	-	-	-
NFS	Kruger[283]	0.77	17.1%	-1.31	76	69	12	99	51	87
NFS	Wong[246]	0.75 (0.67,0.83)	22.8%	-1.455	73	70	13	99	51	86
				0.676	18	96	20	96	63	73

	Study	AU ROC (95%CI)	Prevalence fibrosis	Cut off	Sensitivity %	Specificity %	PPV 5%	NPV fibrosis	PPV 30%	NPV fibrosis
NFS	Kaneda[280]	0.74	27.0%	-	-	-	-	-	-	-
NFS	Ruffillo[301]	0.68	26.8%	-1.455	54	73	9	97	46	78
				0.676	14	100	100	96	100	73
NFS	Wong[309]	0.64 (0.49,0.79)	11.1%	-1.455	39	81	10	96	48	76
				0.676	0	99	0	95	0	70
NFS	Younossi[313]	0.59 (0.47,0.71)	20.3%	-1.722	69	29	4	93	30	69
				-0.833	62	52	6	96	32	75
NFS	Rodriguez[299]	0.08	5.5%	-1.455	100	24	6	100	36	100
				0.676	20	31	1	88	11	48
N-Score	Miyaaki[290]	0.78	17.2%	≤1	84		-	-	-	-
				≥3		82	-	-	-	-
N-Score	Sumida[304]	0.70	10.8%	-	-	-	-	-	-	-
OELF	Rosenberg[235]	0.77 (0.67,1.00)		0.375	89	96	50	99	90	96
				0.462	78	98	67	99	96	91
OELF	Younossi[313]	0.65 (0.53,0.76)	20.3%	-0.523	88	24	5	96	33	81
				1.483	31	93	18	96	64	76
P2/MS	Yu[314]	0.94 (0.84,1.00)	3.0%	49	29	8	2	70	12	22
				95	0	29	0	85	0	40
PAF	Miao[288]	0.84	3.1%	-	-	-	-	-	-	-
Susuki-1	Suzuki[305]	0.88 (0.81,0.96)	25.3%	-	-	-	-	-	-	-

	Study	AU ROC (95%CI)	Prevalence fibrosis	Cut off	Sensitivity %	Specificity %	PPV 5%	NPV fibrosis	PPV 30%	NPV fibrosis
Susuki-2	Suzuki[305]	0.92 (0.85,0.98)	25.3%	-	-	-	-	-	-	-
Tetri-1	Tetri[293]	0.73 (0.68,0.78)	24.6%	-	-	-	-	-	-	-
Tetri-2	Tetri[293]	0.75 (0.70,0.79)	24.6%	-	-	-	-	-	-	-
Tetri-3	Tetri[293]	0.77 (0.73,0.81)	24.6%	-	-	-	-	-	-	-
Tetri-4	Tetri[293]	0.85 (0.82,0.89)	24.6%	-	-	-	-	-	-	-
Younossi-2	Younossi[313]	0.81 (0.70,0.89)	20.3%	0.082	93	35	7	100	38	93
				0.244	87	70	13	99	55	92
Imaging										
ARFI	Yoneda[270]	0.97 (0.93,1.02)	18.5%	1.77 m/sec	100	91	36	100	83	100
ARFI	Palmeri[295]	0.90	29.6%	4.24 kPa	90	90	33	99	79	95
TUE	Yoneda[247]	0.99 (0.97,1.01)	18.5%	9.9 kPA	100	93	42	100	86	100
TUE	Lupsor[285]	0.98 (0.91,1.00)	6.9%	10.4 kPa	100	97	63	100	94	100
TUE	De Ledinghen[248]	0.95 (0.91,0.99)	21.2%	8.7 kPa	84	87	25	98	74	92
TUE	Wong[246]	0.93 (0.89,0.96)	22.8%	7.9 kPa	91	75	16	99	61	95
				8.7 kPa	84	83	20	99	68	92
				9.6 kPa	75	92	33	99	79	90
TUE	Yoneda[312]	0.91	26.9%	8.0 kPa	88	84	23	99	70	94
TUE	Miette[289]	0.91[B]		8 kPa	87	84	21	99	70	94
TUE	Yoneda[247]	0.90	27.8%	9.8 kPa	85	81	18	99	67	93
TUE	Myers[292]	0.90 (0.83,0.98)[XL]	-	-	-	-	-	-	-	-
TUE	Wong[308]	0.88 (0.79,0.97)	16.7%	7.5 kPa	82	71	13	99	56	91

	Study	*AU ROC (95%CI)*	*Prevalence fibrosis*	*Cut off*	*Sensitivity %*	*Specificity %*	*PPV 5%*	*NPV fibrosis*	*PPV 30%*	*NPV fibrosis*
TUE	Petta[297]	0.87	22.6%	8.75 kPa	76	78	16	99	61	89
TUE	Myers[292]	0.87 (0.78,0.97)[M]	-	-	-	-	-	-	-	-
TUE	Freidrich-Rust[275]	0.84 (0.71,0.97)[XL]	-	-	-	-	-	-	-	-
TUE	Myers[291]	0.82 (0.67,0.97)	20.0%	10.3 kPa	70	76	13	98	55	85
TUE	Gaia[276]	0.76 (0.62,0.89)	23.6%	8.0 kPa	65	80	14	97	59	85
TUE	Freidrich-Rust[275]	0.75 (0.55,0.94)[M]	-	-	-	-	-	-	-	-
TUE	Miette[289]	0.74[A]	28.3%	8.7 kPa	46	91	18	97	70	80

APRI aspartate to platelet ratio index; **ALT** alanine aminotransferase; **ARFI** acoustic radiation force impulse; **AST** aspartate aminotransferase; **BMI** body mass index; **CDS** cirrhosis discriminant score; **CK18** cytokeratin-18; **COL-IV** Type IV collagen; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; **GUCI** Goteburg University Cirrhosis Index; **HA** hyaluronic acid; **NAFLD** non-alcoholic fatty liver disease; **NAS** NAFLD Activity Score; **NFS** NAFLD Fibrosis Score; **N-score** Nippon-score; **OELF** Original European Fibrosis panel; **TIMP-1** metallopeptidase inhibitor 1; **TGF-β** transforming growth factor beta; **TUE** transient ultrasound elastography.

2.5 Discussion

2.5.1 Summary of main findings

There have been a large number of studies investigating the diagnostic test accuracy of non-invasive markers for hepatic fibrosis against the reference standard of liver biopsy. Numerous authors have developed differing formulas, largely based on the same components. Very few studies used a development and a validation cohort – the majority have not been replicated in any way.

The studies used were frequently small (26/57 46% with n<100 and 13/57 23% with n>200). Also methodological quality in terms of risk of bias and applicability was often unclear or had definite risks meaning the results needed to be interpreted with caution.

Across all of the markers assessed the AUROCs were widely varied both within and across the markers. Despite this, many assessments did produce an AUROC >0.80 (any fibrosis 15/38 39%, moderate 31/58 53%, significant 58/118 49%) and the proportion above 0.90 improved with fibrosis stage (any fibrosis 3/38 8%, moderate 5/58 8%, significant 23/118 19%). Interpretation with caution is needed. Many did not report confidence intervals around the AUROC, and given the small size of studies and those that were reported, the confidence intervals are wide, often stretching as low as 0.6.

The need to use liver biopsy as the reference standard has limited the generalizability of studies. Populations studied were typically limited to those with a clinical need for biopsy i.e. those with a high level of suspicion of advanced CLD and therefore a high prevalence of liver fibrosis. In some studies the populations were restricted to the severely obese undergoing surgery, a select group who may have different 'normal/abnormal' values of the markers. However, in many general secondary care populations many of the subjects with NAFLD are obese.

Overall, the sensitivity of the markers for diagnosing liver fibrosis of all levels was moderate to poor with a much better specificity. The specificity improved with increasing level of liver fibrosis i.e. the ability to correctly rule out liver fibrosis was good, especially for significant liver fibrosis. However, the PPV was generally poor across all of the markers.

2.5.2 Strengths and weaknesses of the review

In order to facilitate the capture of all reports of non-invasive markers in NAFLD a very wide search strategy and inclusion criteria were successfully employed. A consequential limitation is that the selection of studies and extraction of data of this large number of reports (>11000) was by a single reviewer. The possibility of erroneously discarding a valid report would have been reduced with the use of a second reviewer[118]. Whilst the same criteria were applied to identifying each study the use of a single reviewer risks bias in the study selection through the influence of any existing preferences or opinions[317]. Despite this I have a high level of confidence in the selection of studies given the multiple sources of cross-referencing in study identification including the use of reference and citation searches of included studies.

There is a risk of reporting bias due to the nature of the topic. Only studies finding good or better diagnostic test accuracy tend to be published. It is reassuring that there were some markers with lower AUROCs (5% <0.60) published suggesting that this bias is not as great as it could be. Few markers were replicated and it is unclear if this was attempted and any failure to replicate their findings went un-published. This publication bias is also influenced by the commercial value in identifying a non-invasive marker of liver fibrosis.

Whilst every attempt was made to avoid duplicate publishing of results there is a small possibility of this having occurred. Frequently study results were published as conference abstracts and then later as full research papers. In addition it is small field and the same researchers go on to publish additional data or combine individual markers to form panels and it can be difficult to tease out where the data may relate

to the same patients[318]. To try and avoid this problem this review has clearly marked which reports reflect abstracts and carefully checked author lists to look specifically for the possibility of dual publication.

Another bias is that of language. This review excluded all papers that were not available in the English language, however this proved not to be a problem as the few foreign language papers provided English language abstracts containing sufficient information.

Due to the small numbers in many of the reports is may have been beneficial to undertake a meta-analysis. However, this was not performed because the benefits were unlikely to outweigh the difficulties: time and statistical knowledge, heterogeneity between studies, and many markers had only one or two studies reported.

2.5.3 Agreement/disagreement with other studies

There have been a number of reviews published previously[319-322]. This review is larger, finding more studies for inclusion, than previous systematic reviews and focusses only on liver fibrosis, as opposed to the full NAFLD spectrum. However, despite this the main findings have remained much the same: non-invasive markers have a poor PPV and have improved diagnostic accuracy at higher fibrosis stages. For example, Guha et al[319] and Dowman et al[321] describe 9 different fibrosis markers (vs 49 in this review).

2.5.4 Implications for future research

There are increasing numbers of diagnostic test accuracy studies of non-invasive fibrosis markers continuing to be published, but they are currently adding little to the existing body of evidence. The focus needs to shift to defining and studying the populations of most clinical interest, rather than developing 'more' potential markers in the same select hospital populations. Community-based settings are neglected in the current studies due to the ethical difficulties of performing large scale liver

biopsy. This could be achieved if a non-invasive gold standard could be agreed - for example an imaging modality such as MRE.

2.5.5 Implications for thesis

There are a wide variety of markers that could be considered for use in this thesis for investigating hepatic fibrosis. It is unlikely that any one marker can be used to diagnose hepatic fibrosis given the difficulties discussed above.

The choice of markers to investigate with therefore is affected by several considerations: a) choice of fibrosis stage of interest; b) those markers with a large number of high studies suggestive of good utility; c) those markers which can be utilised easily and acceptably to the study participants, d) efficiency within the studies existing samples and resources, and affordable; e) variety of biological plausibility's of interest; and e) taking into consideration any biases the participants investigated in this thesis may bring to the use of the markers.

Due to the improvement in diagnostic accuracy with increasing liver fibrosis stage and because of the clinical significance for patients this thesis will focus on significant fibrosis/cirrhosis (F3-4). For this stage of fibrosis, APRI, AST:ALT ratio, ELF, NFS and TUE are the most well validated, and will therefore form the focus for the thesis investigations.

Markers such as MRE are both time consuming and expensive and therefore will not be available for use in this thesis.

Many of the pane markers use the same components in their formulas e.g. AST and platelets. For specific analyses the use of similar markers will be limited to reduce the amount of unnecessary statistical testing. Further to this a range of markers based on differing biological plausibility will be investigated e.g. simple non-specific markers, extra-cellular matrix based markers, portal hypertension based markers and imaging.

Some panel markers use and age and the presence of diabetes in their models (e.g. NFS) and as such have the potential to produce repeated high scores in the study given its focus on older people with type 2 diabetes. Markers of this nature will not be used to attempt to diagnose fibrosis in the thesis but will be considered as potential risk factors for other outcomes.

2.6 Conclusions

There is no clear 'best' non-invasive marker of liver fibrosis in patients with NAFLD. At present, the evidence suggests, markers can only be reliably used to exclude the presence of liver fibrosis (high NPV). In addition, markers are better at excluding the presence of advanced fibrosis (Metavir F3-4) than moderate or low grade fibrosis. Given the evidence collected it appears that serum markers will be unlikely to be able to be used to diagnose the presence of liver fibrosis given their low PPV, particularly in low prevalence settings.

CHAPTER 3 Aims and objectives

People with type 2 diabetes are reported to be at greater risk of CLD than the general population. However, little is known of the true burden of CLD in this population in terms of its prevalence and incidence and associated risk factors.

The overall aim of this thesis was to investigate the epidemiology of CLD in older people with type 2 diabetes.

In order to do this, data from the three Edinburgh Type 2 Diabetes Study (ET2DS) data collection phases (baseline, year 1 liver study and year 4 follow-up) was used with a focus on the use of non-invasive markers of liver injury. Non-invasive liver markers were grouped as follows: non-specific liver injury - ALT, AST and GGT; hepatic steatosis - steatosis on USS; steatohepatitis (inflammation/apoptosis) - CK18; liver fibrosis - APRI, AST:ALT ratio, ELF, FIB4 HA, NFS and LSM; and surrogate markers of advanced portal hypertension - platelet count and spleen diameter.

3.1 Research questions

Given the shared mechanisms for both the development of type diabetes and NAFL/D it is believed that NAFL/D is more prevalent amongst those with type 2 diabetes compared to those without, however, it has been difficult to confirm this because of the lack of non-invasive diagnostic techniques. Given the rise in the number of non-invasive markers of NAFLD made available in the past ten years (see Chapter 2) we are now in a possible to start addressing these issues. If the ectopic fat hypothesis is to hold true then it may be expected that patients with more 'severe' type 2 diabetes (a poorer metabolic profile) would have both a higher prevalence of NAFL/D and higher non-markers of NAFL/D.

1. How do the distributions of non-invasive markers of NAFLD (NASH and liver fibrosis) in a community sample of older people with type 2 diabetes compare with a) those in hospital samples of people with type 2 diabetes, and b) those in the general population?

2. Are levels of NASH and NASH-fibrosis (as determined by higher levels of non-invasive markers) higher in patients with a poorer metabolic profile?
3. Are established risk factors for CLD associated with associated with higher levels of non-invasive markers of NAFLD?

If non-invasive markers of hepatic fibrosis are to be useful in the diagnosis of subclinical CLD for further research (and/or clinical practice) clear guidance in their use, with validated cut-offs and diagnostic criteria determined.

4. Do non-invasive markers of hepatic fibrosis agree with each other?

In order determine the clinical need and to inform further research strategies the true burden of CLD in people with type 2 diabetes needs to be determined. In addition prognostic models for the early identification of subclinical CLD and incident CLD are needed. Given the shared aetiology of type 2 diabetes and NAFL/D, it could be hypothesised that risk factors attributable to a poorer metabolic and diabetes profile may be useful in the identification of CLD.

5. What is the prevalence and incidence of advanced clinically significant liver outcomes in older people with type 2 diabetes?
6. Are there any non-invasive markers of CLD or measures of metabolic disease that are associated with prevalent and/or incident clinically significant CLD in older people with type 2 diabetes, that may be useful for prognostic modelling?

It is currently not possible to determine if the relationship between NAFL/D and CVD driven by their shared risk factors, or if NAFL/D 'causes' CVD independently. Investigation of markers related to liver disease, but not known to be related to CVD could help in determining which of these pathways is more likely and aid in the identification of individuals at risk of CVD.

7. Are there any non-invasive markers of CLD associated with prevalent and/or incident CVD in older people with type 2 diabetes, that may be useful for prognostic modelling?

3.2 Aims and objectives

The detailed aims and objectives of this thesis were:

I. To explore the epidemiology of non-invasive markers of NAFLD as potential measures of subclinical CLD in people with type 2 diabetes

   (i) To determine the distribution of non-invasive markers of NAFLD in older people with type 2 diabetes.

   Non-invasive markers will include:

   (i) CK18 (marker of steatohepatitis).

   (ii) APRI, AST:ALT ratio, ELF, FIB4, HA, TE and NFS (markers of liver fibrosis).

   (ii) To investigate the relationship of non-invasive markers of advanced NAFLD with metabolic and CLD risk factors.

   Risk factors will include:

   (i) patient demographics,

   (ii) diabetes history and glycaemic control,

   (iii) metabolic factors,

   (iv) hepatic steatosis, and

   (v) established risk factors for CLD, such as, alcohol, hepatotoxic medication and positive autoantibodies.

   (iii) To determine the level of agreement between five non-invasive markers of liver fibrosis (APRI, AST:ALT ratio, ELF, FIB4 and LSM) in older people with type 2 diabetes.

   (i) Using validated cut-offs to exclude advanced fibrosis; and

   (ii) In the same highest percentile across all marker panels.

II. To determine the frequency of and risk factors for clinically significant CLD in people with type 2 diabetes.

(i) To describe the prevalence (known and unknown) and incidence of CLD amongst older people with type 2 diabetes.

(ii) To determine the association of metabolic and CLD related risk factors with prevalent and incident clinically significant CLD in type 2 diabetes.

Risk factors will include:

(i) patient demographics,

(i) diabetes history and glycaemic control,

(ii) metabolic factors

(iii) established risk factors for CLD,

(iv) markers of liver injury, and

(v) markers of systemic inflammation

III. To determine the importance of CLD as a risk factor (or risk marker) for CV mortality or morbidity in type 2 diabetes.

(i) To determine the association of a range of markers with prevalent and incident CV events and mortality.

Markers will include:

(i) ALT, AST and GGT (markers of non-specific liver injury),

(ii) steatosis on USS,

(iii) CK18 (marker of steatohepatitis)

(iv) AST:ALT ratio and ELF (markers of liver fibrosis), and

(v) platelet count (surrogate marker of portal hypertension).

3.3 Thesis outline

The methods chapter in this thesis describes in detail the methodology of the ET2DS and the data collection and analysis used to investigate liver disease in the ET2DS cohort (Chapter 4).

The subsequent three chapters (Chapters 5, 6 and 7) describe and discuss the results relating to each of the three major aims outlined above.

The thesis concludes with an overall discussion and conclusions (Chapter 8) summarising the main findings of the work, discussing the strengths and limitations of the thesis and outlining future opportunities for research in this area.

CHAPTER 4 Methods

4.1 The ET2DS

The ET2DS is a prospective population based cohort study which commenced recruitment in 2006. Core funded by the Medical Research Council it was conceived primarily to investigate the association between cognitive decline and potentially modifiable risk factors in people with type 2 diabetes. It was later extended to investigate the prevalence of NAFL/D and risk factors associated with its development and progression. In addition, the ET2DS seeks to identify circulating markers and other risk factors which predict the development and progression of symptomatic and asymptomatic micro- and macrovascular disease. It also provides a well phenotyped population sample of subjects for future research.

4.2 The study population

4.2.1 Sampling frame: The Lothian Diabetes Register

The target population was all older people with type 2 diabetes in Lothian, Scotland, and as such the sampling frame used was the Lothian Diabetes Register (LDR). The LDR was fully established in 2001 and records the clinical details of all patients with diabetes in Lothian. Patients are added to the register once a diagnosis of diabetes (WHO criteria) has been confirmed and medical staff can then assign any further classification. Analysis by the Lothian Diabetes Services Advisory Group has found the register contains almost everyone diagnosed with diabetes in Lothian (Prof Sarah H Wild, personal communication).

4.2.2 Definition: type 2 diabetes

For the purpose of the ET2DS, type 2 diabetes was defined as: a diagnosis of type 2 diabetes on the LDR and i) the use of oral antidiabetic agents and/or insulin, or ii)

any subject treated with dietary modification alone whose HbA1c was > 6.5% at the research clinic. All subjects treated with dietary modification alone and with an HbA1c ≤ 6.5% had their medical records reviewed by a consultant Diabetologist to ensure that the diagnosis of diabetes was robust.

The clinical records of individuals who either: (i) started on insulin within one year of diagnosis of diabetes, (ii) reported evidence of pancreatic surgery/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 the greatest risk of misclassification. Any subject in whom it was not possible to confirm a clinical diagnosis of type 2 diabetes by review of hospital and/or general practitioner (GP) records was excluded.

Exclusion criteria included: i) not confirmed type 2 diabetes, ii) non-English speakers (to allow completion of all assessment elements), iii) corrected visual acuity worse than 6/36 for distance vision or unable to read large print text (to allow completion of all assessment elements), iv) unwilling or unable to give consent, and v) physically unable to complete all assessment elements.

4.2.3 Sampling methodology: statistical power and recruitment

Sampling was undertaken with the aim of recruiting 1000 subjects. This would allow 90% power at the two-sided 5% significance level to detect a Pearson correlation coefficient of ≥0.10 between continuous outcomes and independent variables of interest. The sample size was primarily determined for the cognitive function testing.

On the 20th July 2006 there were 9646 patients identified on the LDR aged between 60 and 74 years on 1st August 2006. A total of 5454 potential subjects were randomly selected by sex and 5-year age bands and invited by written invitation to participate by the custodians of the LDR on behalf of the ET2DS.

The detailed recruitment process is shown in Figure 4-1. In brief, 3286 (60.2%) of invitees responded and 1252 (23.0%) expressed an interest in participation. All

subjects expressing interest were invited to the baseline research clinic with 1077 (19.7%) attending. The final study sample consisted of 1066 individuals after a further 11 were excluded due to failure to meet the study inclusion criteria.

To address non-responders to the initial invitation to participate in the study a representativeness analysis was undertaken using data from the LDR to compare those participating with non-responders.

4.3 Data collection

4.3.1 Study phases

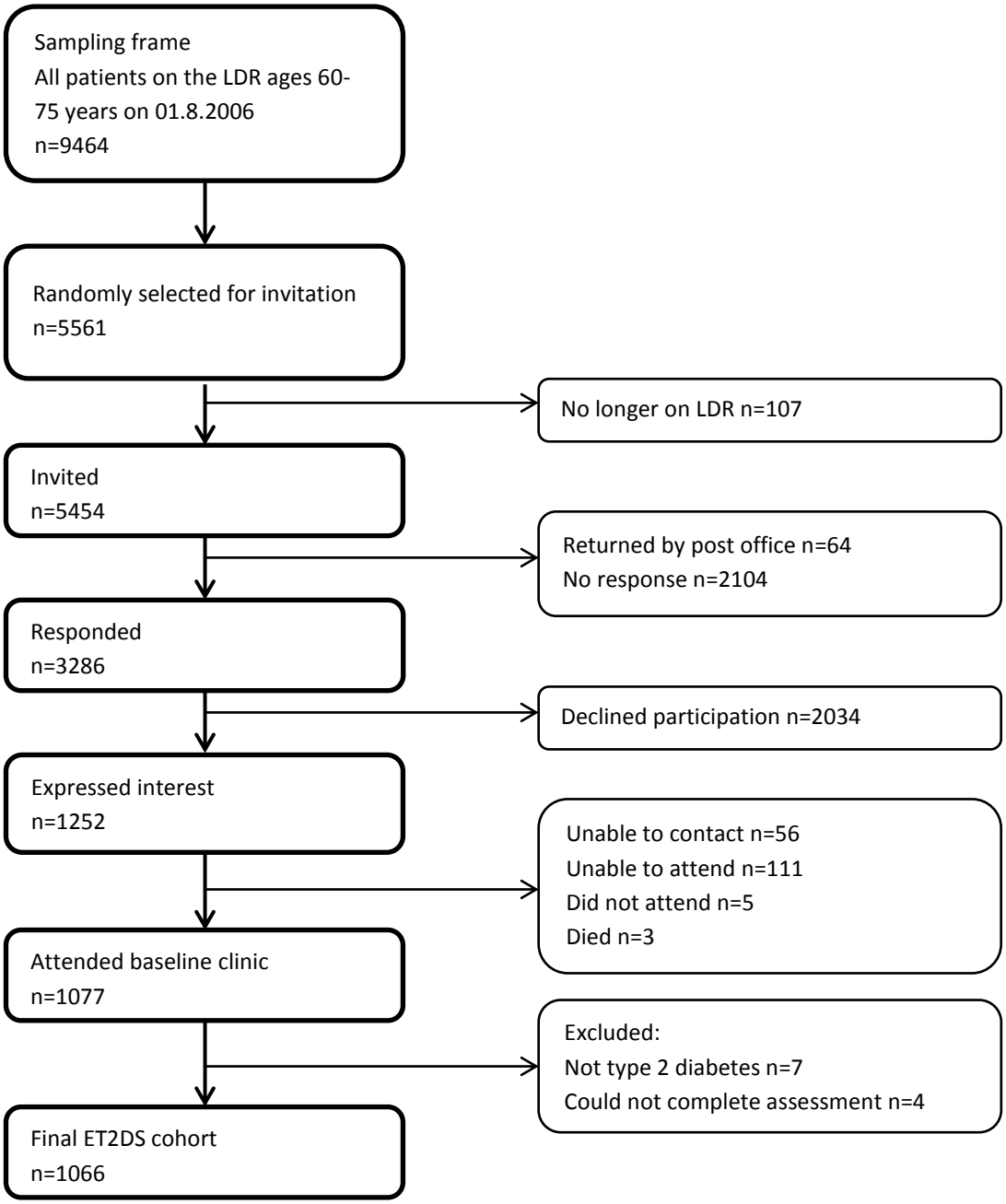
To date the ET2DS has had three phases of data collection in dedicated research clinics. The baseline visits (2006/2007) and initial (year 1) liver sub-study visits (2007/2008) were undertaken prior to my full involvement with the study. I was integrally involved with all aspects of the year 4 follow-up research clinics (2010/2011): design, invitation, data collection, data entry and quality control, and data management. A summary of the data collection at each study phase is shown in Table 4-1.

4.3.2 Research clinics

Research clinics were held at the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh, UK at baseline, year 1 liver sub-study and at follow-up[323,324]. Clinics were run by specially trained research nurses and there were study specific standard operating procedures for each aspect of data collection to ensure accuracy and consistency.

Data were collected on a wide range of variables and only those relevant to this thesis are discussed here. Full details can be found in *Price et al 2008*[323].

Figure 4-1 Edinburgh Type 2 Diabetes Study recruitment and participation flowchart



ET2DS Edinburgh Type 2 Diabetes Study; **LDR** Lothian Diabetes Register

In brief, at baseline and/or initial liver sub-study clinics attendees underwent fasting venous blood sampling, physical examination including (blood pressure measurement and a 12-lead ECG which was subsequently coded using the Minnesota criteria[325] (http://www.sph.umn.edu/epi/ecg/manual/)), and imaging (abdominal USS and TUE). Detailed self-administered questionnaires included standard questions on current medications, alcohol consumption, history of liver disease and CVD and the Edinburgh Claudication and WHO Chest Pain Questionnaires[326,327].

At each research clinic visit patients were asked detailed questions on their diabetes, vascular health and liver disease. In addition, at the LS, patients were asked specifically about joint disease. Patients were also asked to provide a list of current medications and if this was absent subjects were advised that they would be telephoned within the forthcoming week to obtain this information. Medication was subsequently coded using British National Formulary sections. For liver sub-study data, self-reported information on potential hepatotoxic medication use within the previous 6 months was collected and confirmed by review of medical records.

All questionnaires were reviewed with the subject by a research nurse to ensure accurate and complete completion.

Copies of the questionnaires administered at each research clinic are included in Appendix H.

4.3.3 Data linkage

Data linkage was undertaken via the NHS National Services Scotland, Information Services Division, to SMR01 general and acute inpatient discharge records using ICD version 10 and related ICD-9 codes and to Office for Population Censuses and Surveys version 4 codes for CV interventions. Linkage was undertaken following the baseline research clinic and again after the follow-up study (March 2011).

Table 4-1 Summary of data collection in the Edinburgh Type 2 Diabetes Study

Baseline	*Year 1 liver sub-study*	*Year 4 follow-up*	
✓	✓	✓	General questionnaire: current medications, past medical history, alcohol consumption, smoking
	✓	✓	Liver questionnaire: liver disease history, joint disease history, hepatotoxic medication use
✓		✓	Cardiovascular questionnaire: chest pain and claudication scales
✓	✓	✓	Physical examination
✓	✓	✓	Fasting venous blood sample
	✓	✓	Liver USS
	✓	✓	TUE
✓		✓	ECG
✓		✓	Data linkage

ECG electrocardiogram; **USS** ultrasound scan; **TUE** transient ultrasound elestography (Fibroscan®)

The major acute hospitals included in our analyses (Edinburgh Royal Infirmary, Western General Hospital Edinburgh, and St John's Livingstone) all performed above the Scottish average for coding of the main condition causing admission to hospital (www.isdscotland.org/Products-and-Services/Data-Quality/).

4.4 Variable measurement and definitions

For clarity, variables (both risk factor and disease variables) were grouped into categories:

- Demographics: age, sex, socio-economic deprivation.
- Diabetes history: fasting serum glucose, HbA1c, duration of diabetes, treatment type.
- Metabolic factors: BMI, waist circumference, blood pressure, lipids.
- Inflammatory markers: IL6, C-reactive protein (CRP), TNFa.
- Lifestyle risk factors: alcohol and smoking.

- Non-invasive liver markers: non-specific liver injury, inflammation and apoptosis, liver fibrosis, surrogates of portal hypertension
- NAFL/D and CLD outcomes.
- CV events.

4.4.1 Demographics

Date of birth (for age) and sex of subjects was obtained from the self-report questionnaire and assessed against information on the LDR, with discrepancies being resolved by referring to clinic records.

Socio-economic status was measured using the Scottish Index of Multiple Deprivation (SIMD) 2006 converted from patient home postcodes at baseline (see http://openscotland.gov.uk/Topics/Statistics/SIMD/FAQs#lookups) and defined as quintiles.

4.4.2 Diabetes history

Duration of diabetes was calculated from the date of diagnosis provided by the subject on the self-report questionnaire. This was also defined as a binary categorical variable: <5 years and ≥5 years.

Diabetes treatment type was revised following each research clinic attendance from the self-report questionnaire and medication lists. Data from both diabetes specific questions and the general medication question was used to define treatment type as: i) diet controlled, ii) oral anti-hyperglycaemic agent (OAHA) only, and iii) insulin +/- OAHA. Additionally OAHA were sub-categorised into i) metformin, ii) sulphonylureas, and iii) thiazolidinediones.

Serum fasting glucose and HbA1c were measured on the fasting venous blood samples and analysed using a Vitros Fusion chemistry system (Ortho Clinical Diagnostics, Bucks, UK) at the Western General Hospital (Edinburgh, UK).

4.4.3 Metabolic factors

Height was measured using a wall-mounted vertical rule standing height was measured to the nearest mm, standing without shoes. Weight was measured using SECA 761 electronic weighing scales to the nearest 0.1 kg without outdoor clothing or shoes. BMI was subsequently calculated height (in m)/weight (in kg)^2.

Waist circumference was measured using a non-expandable tape measure twice and the average of two readings taken to the nearest 0.5 cm was taken as the final measurement. Measurements were made at the level midway between the lower rib margin and the iliac crest, with the subject standing with their feet 30 cm apart and with their hands by their sides, during exhalation.

Systolic and diastolic blood pressures were measured using a standard stethoscope and an aneroid, 6 inch dial, desk standing sphygmomanometer (Acceson™, AC Cossor & Son (Surgical) Ltd, Harlow, UK) in the supine position in the right arm to the nearest 2 mmHg.

Lipids (total cholesterol, HDL-cholesterol and TG) were measured using the fasting venous blood sample and analysed using a Vitros Fusion chemistry system (Ortho Clinical Diagnostics, Bucks, UK) at the Western General Hospital (Edinburgh, UK).

4.4.4 Inflammatory markers

CRP was measured using an immunonephelometric assay, and IL-6 and TNF-α were measured using the ELISA system (R&D Systems, Oxon, UK), all at the Glasgow Royal Infirmary, UK.

4.4.5 Lifestyle risk factors

Average alcohol intake per week over the previous year and history of alcohol excess were determined from two questions in the self-completion questionnaire, adapted from the AUDIT-C screening tool[328]: "How often did you have a drink containing alcohol in the past year?"(a drink was considered to be one and a half alcohol units);

and “How many drinks did you have on a typical day when you were drinking in the last year?”.

Average alcohol units during a drinking opportunity = 1.5 * “How many drinks did you have on a typical day when you were drinking in the last year?

Average alcohol units per week = average units during a drinking opportunity * average number of drinking days per week (from “How often did you have a drink containing alcohol in the past year?”). Creating a continuous variable.

Alcohol excess was defined according to established criteria as alcohol intake >14 units/week (female) or >21 units/week (male)[70], or subject self-report of current/previous alcohol excess. Resulting in a binary yes/no variable.

Patients were asked if they were a current smoker, had ever been a regular smoker and questions about how much and what they smoked.

Smoking was then defined categorically as current, ever or never.

4.4.6 Non-invasive liver markers

Non-specific liver injury

Plasma liver enzymes (ALT, AST and GGT) were measured using the fasting venous blood sample and analysed using a Vitros Fusion chemistry system (Ortho Clinical Diagnostics, Bucks, UK) at the Western General Hospital (Edinburgh, UK).

Abnormal liver enzymes were defined as: i) abnormal - greater than the upper limit of normal (ULN) – ALT >50U/L, AST >45 U/L, GGT >55U/L; ii) highly abnormal - greater than twice the ULN for ALT, AST, GGT; and iii) greater than recently proposed sex specific cut-offs for ALT – males >30U/L, females >19U/L[329].

Hepatic steatosis

Presence of liver fat (hepatic steatosis) was determined by abdominal USS using a Sonoline Elegra Ultrasound Imaging System (Siemens Medical Systems Inc, Washington, USA), software version 6, with a 3.5 MHz transducer. The same

sonographer, blinded to the subjects' clinical history, undertook all scanning and grading. The liver was graded for markers of hepatic steatosis using established criteria[165]. Subjects were given an overall hepatic steatosis grade based on a subjective measurement of the severity of steatosis: normal, mild, moderate or severe. In a subset, steatosis was validated using magnetic resonance spectroscopy[330] following which grades were condensed to: "normal" or "significant steatosis". Radiological signs of cirrhosis were also noted and spleen size was measured in cm.

Non-alcoholic steatohepatitis (inflammation and apoptosis)

CK18 was measured on samples collected at the research clinic and stored at -80^{o}c and analysed using the M30-Apoptosense® ELISA (PEVIVA AB, Stockholm, Sweden) at the Biomedical Research Unit laboratory (University of Nottingham, UK).

Liver fibrosis

A wide range of markers of fibrosis were measured (APRI, AST:ALT ratio, ELF, FIB4, NFS and TUE).

APRI calculated as [[AST(U/L)/ULN]/platelets(x10^{9}/L)]x100[208].

AST:ALT ratio calculated as AST(U/L)/ALT(U/L).

ELF calculated as 2.588 + (ln(HA)*(ln(P3NP)*0.775) + (ln(TIMP1)*0.494)[236].

FIB4 calculated as [age(years)*AST(U/L)]/[platelets(x10^{9}/L)*$\sqrt{}$AST(U/L)][331]

NFS calculated as -1.675+0.037*age(years)+0.094*BMI(kg/m^{2})+1.13*IFG/diabetes (yes=1, no=0)+0.99*AST:ALT ratio-0.013*platelet count(×10^{9}/L)-0.66*albumin (g/dL)[243]

ELF was measured on samples collected at the research clinic and stored at -80^{o}c and analysed using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics Inc, New York, USA) at the iQur laboratory (London, UK).

One dimensional TUE was performed using a FibroScan® (Echosens, Paris, France) machine. A single operator was formally trained by Echosens personnel prior to commencement of the study. Initial ultrasound assessment allowed measurement of the skin-capsule distance. For skin-capsule distances <2.5cm the M probe was used, for ≥2.5cm the XL probe was used in accordance with recommended standard Fibroscan operating procedures.

The TUE probe was placed in an intercostal space overlying the liver with the patient in the supine position. Using ultrasound to guide positioning, an area of the liver that was at least 6cm deep and free from large vessels was selected for investigation. The area measured was between 25mm-65mm below the surface of the skin for the M probe and 35mm-75mm for the XL probe. The operator aimed to obtain ten valid LSMs. All scans were undertaken in the fasting state. Every six months the probes were serviced and calibrated.

Surrogates of portal hypertension

Platelets were measured as part of the full blood count. This was also defined as a binary categorical variable: <150 $x10^9$/L and ≥150 $x10^9$/L.

Spleen diameter was measured during the abdominal USS. This was also defined as a binary categorical variable: >13cm and ≤13 cm.

4.4.7 Non-alcoholic fatty liver /disease and chronic liver disease outcomes

NAFL/D was defined as the presence of hepatic steatosis on USS without alcohol excess or use of hepatotoxic medication and a negative liver screen[61].

Alcohol excess was as defined above (Section 4.4.5). Hepatotoxic medication use was defined as the use of non-topical glucocorticoids for >2 weeks, isoniazid, methotrexate, amiodarone, or tamoxifen within the 6 months prior to USS. A positive liver screening included any of positive autoantibodies (any of anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-mitochondrial antibody (AMA)), ferritin >1000 ng/mL, alpha-feto protein >6 ng/mL, or positive hepatitis B

or C serology). Clinically significant positive immunology titres were defined as ASmA titre >1:160 or AMA titre >1:40[332].

'Clinical' CLD and clinically significant CLD (prevalent and incident) were identified from multiple sources. CLD was defined as a clinician diagnosis of any aetiology and stage of CLD. Definite clinically significant CLD was a composite outcome defined as a diagnosis of cirrhosis, HCC (with confirmatory radiology) or gastro-oesophageal varices (with confirmatory endoscopy) recorded in the patients' medical records.

Research study referral criteria were designed in conjunction with an experienced consultant Hepatologist for incident abnormalities found during the year 1 clinic which suggested that a patient may be at high risk of clinically significant CLD. The referral criteria were any of: routine liver enzyme tests above the laboratory ULN (ALT >50 U/L, AST >45 U/L, GGT >55 U/L, ALP >125 U/L); AST:ALT ratio >1; a positive liver screen (described above), hyaluronic acid >100 microg/L (in the absence of known joint disease),spleen >13cm (in the absence of known haematological cause), platelets <150 $x10^9$/L (in the absence of known haematological cause) or suspected cirrhosis on USS.

In addition to clinical examination, prevalent and incident clinically significant CLD was identified using a two-stage process across multiple sources. Stage one: *possible* cases were identified from year 1 liver sub-study and year 4 follow-up clinic patient self-reported questionnaires; data linkage to ICD-9/10 and Office for Population Censuses and Surveys coding within SMR01 general and acute inpatient discharge records (at NHS National Services Scotland, Information Services Division) in July 2008 and July 2011; non-attenders at the year 4 follow-up clinic were sent a postal questionnaire and if no response was received a modified questionnaire was sent to the GP. Stage two: *definite* cases were confirmed through the review of all patients' electronic secondary care medical records (TrakCare, InterSystems Corp., Cambridge, USA) until 31 December 2013 in order to verify all possible diagnoses and to identify any additional CLD. No patient had an ICD-10 code (cirrhosis

K70.3, K74.3, K74.4, K74.5, K74.6; HCC C22.0; gastro-oesophageal varices I98.2) without confirmation in their medical records.

4.4.8 Cardiovascular events

Information on CV events at baseline and at follow-up clinics was collected from multiple sources, including patient and/or GP completed questionnaires, 12-lead ECG, and linkage to hospital discharge and death certification data. A fatal or non-fatal CV event was recorded if pre-determined criteria based on the multiple data sources were met.

Myocardial Infarction: (1) ICD-10 code for myocardial infarction on discharge/death record, plus either subject report of a doctor diagnosis of myocardial infarction, positive WHO chest pain questionnaire for myocardial infarction, report of myocardial infarction on GP questionnaire or new ECG codes for myocardial infarction or (2) clinical criteria for myocardial infarction met following scrutiny of hospital and/or GP notes. Angina: (1) ICD-10 code for angina as primary diagnosis on discharge record, or (2) at least 2 of (a) subject report of a doctor diagnosis (self-report) of angina or of starting angina medication, (b) ECG codes for ischaemia, and (c) positive WHO chest pain questionnaire, or (3) clinical diagnosis of angina on scrutiny of hospital notes. Stroke: (1) ICD-10 code for stroke as discharge/death record, or (2) clinical criteria for stroke met on scrutiny of clinical notes in subjects with either self-report of stroke or with non-primary ICD-10 hospital discharge/death code for stroke. Transient ischaemic attack: (1) ICD-10 code for transient ischaemic attack on discharge record, or (2) clinical criteria for transient ischaemic attack met on scrutiny of clinical notes in subjects with either self-report of stroke or with non-primary ICD-10 hospital discharge code for stroke or transient ischaemic attack. Coronary intervention: (1) Office for Population Censuses and Surveys -4 code for coronary intervention on discharge record. Intermittent claudication: (1) ICD-10 code for intermittent claudication on discharge record, or (2) clinical criteria for intermittent claudication met on scrutiny of clinical notes in subjects with either self-report of intermittent claudication, or (3) positive Edinburgh Claudication Questionnaire. Peripheral vascular intervention: (1) Office for Population Censuses

and Surveys -4 code for peripheral intervention on discharge record. Carotid endarterectomy: (1) Office for Population Censuses and Surveys -4 code for carotid endarterectomy on discharge record.

Coronary artery disease (CAD) was defined as any of myocardial infarction, angina or coronary intervention and CVD as any CAD event or any of peripheral vascular intervention, stroke, transient ischaemic attack, and carotid endarterectomy.

4.5 Ethical approval

Initial ethical approval for the ET2DS was granted by the Lothian Medical Research Ethics Committee, both for the baseline study and then the liver sub-study. I applied for an amendment to existing approvals for the follow-up study and this was subsequently granted.

Permission to access the LDR was given by the Lothian Diabetes Services Advisory Group and the Caldicott Guardian for NHS Lothian.

Written informed consent was obtained from all subjects on attendance at each phase of the study.

4.6 Data management

4.6.1 Data security

Data were inputted into a master database (Microsoft Access 2003/2010, Microsoft Corporation, Washington, USA) and was held and backed up securely on a dedicated university server requiring both electronic permission to access the storage drive and password access to the database. Paper records were stored in secured filing cabinets within a locked office with only authorised access allowed.

4.6.2 Quality assurance

All paper records were manually entered into the database. At baseline clinic records were double entered onto the database and checked for accuracy. Discrepancies were settled by discussion. At follow-up 10% (n=80) randomly selected records were double entered and checked for accuracy. An overall error rate of 0.017 was calculated, reduced to 0.010 for important errors (i.e. deemed to have potential to affect onward analysis).

Laboratory data was either manually entered from paper reports or electronically entered from files provided by participating laboratories.

4.7 Data cleaning and analysis

Data were analysed using SPSS v19.0 (SPSS Inc., Illinois, USA) and/or R.

4.7.1 Data cleaning

Following data entry, descriptive analyses were run on all measurement data. These distributions were examined for outliers (any result felt to be erroneous based on medical knowledge and accepted laboratory limits) and for missing values. For each outlier the original paper records were consulted and corrections made as appropriate. For any persisting outliers those felt to be implausible were deleted and treated as missing. Plausible but outlying data was allowed to remain and its use decided upon dependant on the individual research question. For the results reported in Chapters 5, 6 and 7 plausible outliers were retained as they were felt to be relevant to the research aims and objectives.

4.7.2 Missing data

Both the ET2DS as a whole and variables included in the dataset used for this thesis were subject to missing data. A number of study participants chose not to attend research clinics for the initial liver sub-study and/or follow-up, for some aspects of data collection subjects were unable to complete assessments at a given research

clinic due to physical inability, and for some specific variables, measurements on a patients biological sample were not obtained (e.g. due to limited sample being available). The degree of missing data by individual variable is described in section 5.1.2 in this thesis. The general approach to missing data throughout the thesis is described here.

There are a number of ways of addressing missingness due to non-attendance at a phase of a study and item missingness due to failure to complete a single component of the study. A common way to address missingness is to restrict the analysis to subjects with no missing values in the specific analysis variables (available-case analysis) and in the case of multiple analyses exclude cases with any of the necessary data missing (complete-case or list-wise analysis). If the missing data is missing completely at random this may be a valid approach, however there may be a systematic reason why data elements are missing resulting in a biased effect estimate[333]. Also the combined exclusions from such an approach can lead to a substantial loss of the original sample and reduce the original power and precision intended. For the purpose of this thesis, where there were small amounts of missing data (<5%) assumed to be missing completely at random available–case analysis was used.

It is not possible to determine from observed data alone whether data are missing at random (systematic difference between the missing values and the observed values can be explained by differences in observed data) and missing not at random (even after the observed data are taken into account, systematic differences remain between the missing values and the observed values)[334]. In order to assess the potential for missing not at random where variables had a large quantity of missing data (>5%) a) the method of obtaining the data was considered for the introduction of bias and b) systematic differences in the populations with and without the variable of interest was explored.

The most common approaches to dealing with large quantities of missing completely at random data involve including individuals with incomplete data to maintain statistical power. Options include: including partially available variables (random

effects models in longitudinal analysis), weighting the analysis to allow for the missing data[335,336], and modelling the reasons for missing data and the associations of interest (maximum likelihood estimation)[337], or through the creating of multiple datasets with imputed replacements for the missing data points (multiple imputation)[338].

Based on the statistical practicalities and types of analyses planned, where there were large quantities of missing completely at random or missing at random data in this thesis, the usefulness of multiple imputation was trialled and compared with available-case analysis (sensitivity analysis) in an attempt to minimise the effects of the missing data on the study's power (i.e. maximise the inferential validity, not to recover missing values).

Multiple imputation involves the creation of multiple plausible (complete) datasets. The purpose of multiple datasets is to reflect the uncertainty due to imputation. Each dataset is then analysed separately and the point estimates and the estimated standard errors combined.

It should be noted that multiple imputation is not without its own limitations and a number of practical problems. Either multiple datasets including all dependant and independent variables under consideration in the study need to be created or separate imputed datasets are required for each research question. If all of the variables of interest both dependant and independent are not included the dataset is biased and the strength of any associations will be weakened[339]. Non-normally distributed variables require to be transformed and then the imputed dataset transformed back to insure that a skewed variable does not develop implausible or impossible values (although in some techniques this can be addressed by putting limits on the imputed values). In order to maintain the missing completely at random assumption all variables in the planned analysis must be included and all variables associated with the missing variable and all variables influencing the process causing the missing data, even if they are not of interest in the substantive analysis[340]. Multiple imputation can be labour intensive for both the operator and the computer. There are a wide range of different imputational methodologies and the suggested number of imputed datasets

varies widely. For variables with large quantities of data missing completely at random multiple imputation by chained equations[341] was undertaken to allow sensitivity analyses of analyses with the missing data to be performed.

4.7.3 Statistical analysis

Distributions of liver markers

Hypothesis: Type 2 diabetes and NAFL/D share an underlying pathological mechanism (the ectopic fat hypothesis) therefore it may be expected that patients with more 'severe' type 2 diabetes (a poorer metabolic profile) would have both a higher non-markers of NAFL/D.

To explore the distribution of markers of sub-clinical liver disease, data from the year 1 liver sub-study was used, with the exception of LSM which was only measured at the follow-up clinic.

The associations of each of the markers of steatohepatitis (CK18) and liver fibrosis (APRI, AST:ALT ratio, ELF, FIB4, HA, NFS and LSM) with the following were examined, (i) duration of diabetes and diabetes treatment, (ii) metabolic variables (total cholesterol, triglycerides, fasting glucose, HbA1c, BMI and waist circumference), (iii) steatosis on USS and, (iv) established risk factors for CLD (alcohol excess, hepatotoxic medication, positive immunology). Analyses were undertaken on (i) all subjects, (ii) subjects with steatosis (defined as the presence of steatosis on USS) and, (iii) subjects with NAFL (defined as the presence of steatosis on ultrasound without alcohol excess, use of hepatotoxic medication or raised autoantibodies). The influence of CKD (defined as estimated glomerular filtration rate ,eGFR <60) and arthritis (patient reported history of osteoarthritis, rheumatoid arthritis, scleroderma or any other joint disease) on circulating biomarker level was also assessed by analysing the prevalence of these conditions in the highest and lowest quintiles of each biomarker.

Univariate analysis on the association between liver markers and potential risk factors was undertaken using Pearson's correlation and ANOVA adjusting for age

and sex. Multivariable analysis was undertaken using linear regression both unadjusted and fully adjusted for age, sex, and established hepatic risk factors. All continuous variables were assessed for approximation to the normal distribution. Where necessary, to address skewed data, CK18, APRI and HA were transformed on the logarithmic scale for analysis. The maximum number available was used for each analysis. A sensitivity analysis addressing missing CK18 and ELF data was performed using multiple imputation by chained equations[341]. Data were considered to be missing completely at random as they were missing due to technical problems or insufficient stored sample. Analysis was undertaken on both the whole population without pre-diagnosed liver disease and additionally in those with NAFL/D (defined as the presence of hepatic steatosis on USS without alcohol excess, use of hepatotoxic medication or abnormal liver screen).

Liver fibrosis marker agreement

Hypothesis: Hepatic fibrosis is a histologically advanced stage of CLD liver disease. Validated non-invasive measures of hepatic fibrosis would be expected to agree with each other as they are all measuring the same underlying pathological stage.

The agreement between different markers of liver fibrosis was assessed - the five markers compared were APRI, AST:ALT ratio, ELF, FIB4 and LSM, all measured at year 4 follow-up. These markers where chosen to include a range of different test rationales/measures (AST:ALT ratio – hepatocyte damage, APRI – hepatocyte damage and portal hypertension, ELF- extracellular matrix composition, FIB4 - hepatocyte damage and portal hypertension, and LSM - imaging) whilst avoiding bias from within the study population (e.g. through the inclusion of age or BMI).

Validated cut-offs to reliably exclude advanced fibrosis (≥ Metavir F3) in NAFLD were determined from the literature aiming to achieve negative predictive values (NPVs) 90-95%:

i) APRI=1.0, specificity (spec.) 89%, NPV 84%[208,228];

ii) AST/ALT=1.0, spec. 90%, NPV 89%[228];

iii) ELF=10.358, spec. 94%, NPV 90%[236];

iv) FIB4=1.30, spec. 65%, NPV 95%[228,331]; and

v) LSM=8.7, spec. 83.2%, NPV 94.6%[246].

Since these threshold levels described above cannot reliably be extrapolated from the predominantly secondary care settings in which they were validated to a general population setting, I also assessed agreement between markers using the same highest percentile across all marker panels. Prior studies suggest that the prevalence of advanced fibrosis in type 2 diabetes patients attending outpatient clinics is at least 2-6% in all patients and 7-12% in those with NAFL/D[159,161,163]. As a result I estimated that the underlying prevalence of significant liver fibrosis in the whole ET2DS cohort might be in the region of 5% and around 10% in those with NAFL/D. I therefore compared the top 5% (and 10%) of scores for each marker for agreement in the entire cohort (and in those with NAFL/D respectively).

Correlation between markers was analysed after standardisation to Z-scores, and adjusted for age and sex. Cronbach's alpha was used to examine the inter marker agreement (using standardised Z-scores). Student's t-test or the Mann-Whitney U test were used to compare means and Chi-squared test to compare proportions.

Individual 2x2 tables were calculated for the absence/presence of probable fibrosis (based on percentiles) for each pair of markers. Due to the difficulties interpreting markers of total agreement[342] (e.g. kappa statistics), I calculated positive agreement (agreement on the presence of fibrosis by both markers) and negative agreement (agreement on the absence of fibrosis by both markers)[343] as shown in Figure 4-2.

Figure 4-2 Agreement

		Marker 1 Fibrosis present	Marker 1 Fibrosis absent	Total
Marker 2	Fibrosis present	a	b	a+b
Marker 2	Fibrosis absent	c	d	c+d
	Total	a+c	b+d	a+b+c+d

Positive agreement =2a/(2a+b+c)

Negative agreement =2d/(b+c+2d)

Frequency of and risk factors for clinically significant chronic liver disease

Hypothesis: Given the shared aetiology of type 2 diabetes and NAFL/D, it would be expected that the prevalence and incidence of clinically relevant CLD would be higher in those with type 2 diabetes than in the general population and that risk factors attributable to a poorer metabolic and diabetes profile may be useful in the identification of CLD.

In order to measure clinically significant CLD, the outcomes of the research clinic investigations and specialist Hepatology referral process were reported for all subjects without known prevalent clinically significant CLD. In addition, for all subjects without prevalent clinically significant CLD following the year 1 investigations, rates of incident clinically significant CLD (/1000 person-years) during the follow-up period were calculated for those referred to Hepatology clinic, those seen in Hepatology clinic, those with abnormal liver enzymes, and for those with hepatic steatosis. Negative binomial regression was used to calculate incidence rate ratios (IRR).

Known prevalent clinically significant CLD was defined as: the number of patients with known clinically significant CLD at the liver sub-study research clinic visit (numerator) divided by the number of all patients attending the liver sub-study (denominator).

Unknown prevalent clinically significant CLD was defined as: the number of patients with clinically significant CLD identified within 12 months as a direct result of the liver sub-study research clinic visit (numerator) divided by the number of all patients without clinically significant CLD after the liver sub-study (denominator).

Incident clinically significant CLD was defined as: the number of patients with clinically significant CLD identified subsequent to the liver sub-study research clinic visit, excluding cases identified as unknown prevalent (numerator) divided by the number of person-years without the development of clinically significant CLD (ie total time for all subjects without clinically significant CLD from attendance to the first of development of clinically significant CLD, death, or 31 December 2013). Incidence was presented as a rate per 1000 person-years.

Exploratory analysis of potential risk factors and markers associated with the development of clinically significant CLD was under taken using : (i) patient characteristics (age, sex and SIMD quintile), ii) diabetes history (duration of diabetes categorised as $<$ or $\geq$ 5 years; diabetes treatment categorised as diet-controlled, OAHA, insulin, or insulin +/- OAHA; fasting glucose; HbA1c), (iii) metabolic variables (total cholesterol, triglycerides, BMI calculated as weight (kg)/height $(m)^2$ and systolic blood pressure), (iv) established risk factors for CLD (alcohol excess, hepatotoxic medication, positive autoantibodies), (v) markers of liver injury, including those measuring non-specific liver injury (plasma liver enzymes), steatosis (USS), steatohepatitis (CK18), surrogates of advanced portal hypertension (platelet count, spleen size), and liver fibrosis (APRI, AST:ALT ratio, ELF, FIB-4, HA, NFS), and (vi) markers of systemic inflammation (CRP, IL-6 and TNF-α)

For continuous variables mean (sd) or median (IQR) were calculated and for categorical variables % (n) reported for those patients with/out unknown prevalent and incident clinically significant CLD. Cox proportional hazards regression was used to determine the risk (HR) of each risk factor associated with the development of incident clinically significant CLD.

Association of liver markers with cardiovascular disease

Hypothesis: If NAFL/D 'causes' CVD through the atherogenic liver hypothesis, as opposed to the relationship between NAFL/D and CVD being driven by their shared risk factors, it would be expected that markers of the full spectrum of NAFL/D would be related to the development of CVD.

In order to maximise the follow-up time available for CV events to occur the markers collected at the baseline clinic were used where available. The association of markers of non-specific liver injury (ALT, AST, GGT measured at baseline), hepatic steatosis (steatosis on USS measured at year 1), steaohepatitis (CK18 measured at year 1), liver fibrosis (APRI, AST:ALT ratio, FIB4 and NFS measured at baseline, and ELF measured at year 1), and portal hypertension (platelets measured at baseline) with prevalent and incident CV events and mortality were investigated.

Individual cardiovascular events were determined from multiple sources as described in section 4.4.8.

The primary outcome measures were all prevalent CV events and all incident CV events. The secondary outcome measures were prevalent and incident CAD events. Fatal and non-fatal events were combined for analysis. Prevalent CAD and CVD were taken as any event prior to the baseline/liver sub-study research clinic attendance. Incident CAD and CVD were taken as defined as any new event occurring between baseline/liver sub-study research clinic attendance and the end of August 2011, for both non-fatal and fatal events.

The follow-up time for each individual for incident disease was from the date of the baseline/liver sub-study research clinic attendance until the first of: CV event, death or end of August 2011.

Analysis was undertaken using a listwise approach for three scenarios – measurements taken at baseline (ALT, APRI, AST, AST:ALT ratio, FIB4, GGT, NFS and platelets), measurements taken at the initial liver sub-study clinic (CK18 and steatosis on USS) and ELF.

Univariate analysis with normal continuous variables was analysed using Student's t-test (ALT, AST, AST:ALT ratio, ELF FIB4, NFS and platelets), non-normal continuous variables (APRI, CK18 and GGT) using the Mann-Whitney U test and categorical variables (steatosis) using the ChiSq test was examined for both the presence of prevalent and incident CVD and CAD.

Logistic regression for the association with prevalent CVD and CAD and Cox proportional hazards regression for the association with incident CVD and CAD was undertaken for all markers of liver injury. Both were performed unadjusted, adjusted for age and sex and additionally adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, BMI, systolic blood pressure (sBP), diastolic blood pressure (dBP), HbA1c, HDL cholesterol, total cholesterol and eGFR. Analysis of incident disease was also adjusted for baseline/year 1 history of prevalent CVD/CAD.

A sensitivity analysis of the incident CV events was undertaken by excluding all subjects with prevalent CVD at baseline and the Cox regression then repeated.

4.7.4 Cut point for statistical significance

Conventional statistical cut-offs of $p<0.05$, $p<0.01$ and $p<0.001$ are highlighted within the results with respect to rejecting the null hypothesis, ie a <5%, <1% and <0.1% chance (respectively) that the null hypothesis is rejected when is in in fact true (type I error).

This risk of obtaining a statistically significant result increases as the number of statistical tests performed increases e.g. 1 test 5% chance, 10 tests = 40% chance ($1-[1-0.5]^{10}$), 50 tests = 93% chance ($1-[1-0.5]^{50}$).

Some would argue the necessity adjust analyses to take this into account. Most commonly the Bonferroni correction is applied whereby the significance level at which the null hypothesis is rejected is adjusted to reflect the increased risk from multiple testing – the original significance level is divided by the number of

individual tests e.g. 1 test = 0.05 (0.05/1), 10 tests = 0.005 (0.05/10), 50 tests = 0.001 (0.05/50). There are several problems with this approach[344]. Firstly, the Bonferroni correction increases the risk of failing to reject a false null hypothesis (type II error). Secondly, is the decision as to how to count the number of analyses to which to apply the correction. In thesis would it be all the tests pertaining to a single research question, to a section, to a chapter, across the whole thesis, to the authors total involvement in the ET2DS? In addition, some of the analyses and decisions in this thesis are based on the results of earlier analyses (dependant) so it is not possible to exactly define the number of tests *a priori*.

In order to attempt to address these issues whilst conventional statistical cut-offs of are highlighted within the results (and when text refers to non/significant results it a 0.05 cut off), where possible exact p values and 95% confidence intervals are reported to allow the reader to make a context specific judgement. Given that much of the work within this thesis is exploratory and hypothesis generating the number of tests performed will inevitably be large, however, informative results can then be taken forward and investigated in more focused studies.

CHAPTER 5 Results I: non-invasive markers of steatohepatitis and liver fibrosis in older people with type 2 diabetes – distributions, associated risk factors and levels of agreement

Given the potential burden caused by both asymptomatic and symptomatic clinically significant CLD in the general diabetic population, there is a need to identify non-invasive methods of detecting asymptomatic stages of the condition in adults with diabetes, as indeed is the case for other high risk sub-groups within the general population.

In order to investigate a number of potentially useful non-invasive of liver disease in a diabetic population, in this chapter I have:

(i) described the study population used in this thesis (numbers, representativeness, patient characteristics),

(ii) described the variables included in subsequent analyses (in terms of missingness and statistical distributions),

(iii) determined the distribution (by gender, steatosis and NAFL/D), and factors associated with altered levels of, a range of potential non-invasive markers of steatohepatitis (CK18) and liver fibrosis (APRI, AST:ALT ratio, ELF, HA, FIB4, NFS and LSM),

(iv) determined the level of agreement between five potential markers of liver fibrosis (APRI, AST:ALT ratio, ELF, FIB4 and LSM):

   a. using validated cut-offs to exclude advanced fibrosis; and

   b. in the same highest percentile across all marker panels.

5.1 Study population

The ET2DS baseline cohort comprised of 1066 individuals. All patients still alive were invited to the liver sub-study at 1-year, with 939 subjects attending. Again all surviving subjects were invited to the 4-year follow-up, with 831 subjects attending. By follow-up 88 subjects were deceased. Full details of non-attendance at the research clinics for each phase of the study are shown in Figure 5-1 and Figure 5-2.

5.1.1 Representativeness

At baseline the ET2DS research team undertook a representativeness analysis[321] of those recruited into the study (n=1066) versus those invited from the LDR but not participating (n=4388). I repeated a similar analysis for subjects attending the liver sub-study one year after baseline (versus ET2DS participants not attending the liver sub-study) and again for subjects attending the follow-up clinic four years after baseline (versus participants not attending the follow-up clinic. It should be noted that participants not attending the liver sub-study or follow-up research clinics were not lost to the study for all outcomes as they were still able to complete a questionnaire (or their GP on their behalf) and they remained subject to record linkage.

Table 5-1 compares the characteristics of responders with non-responders at baseline and the baseline characteristics of research clinic attenders with non-attenders at each subsequent phase of the study.

Figure 5-1 Edinburgh Type 2 Diabetes Study liver study participation flowchart

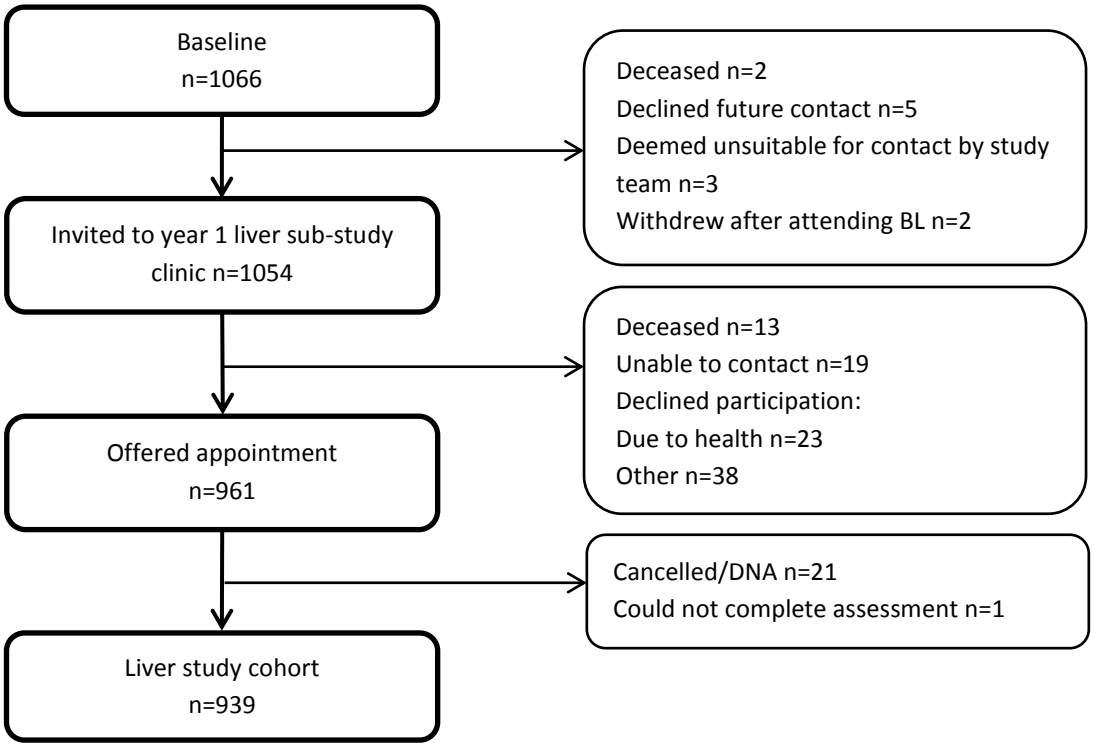
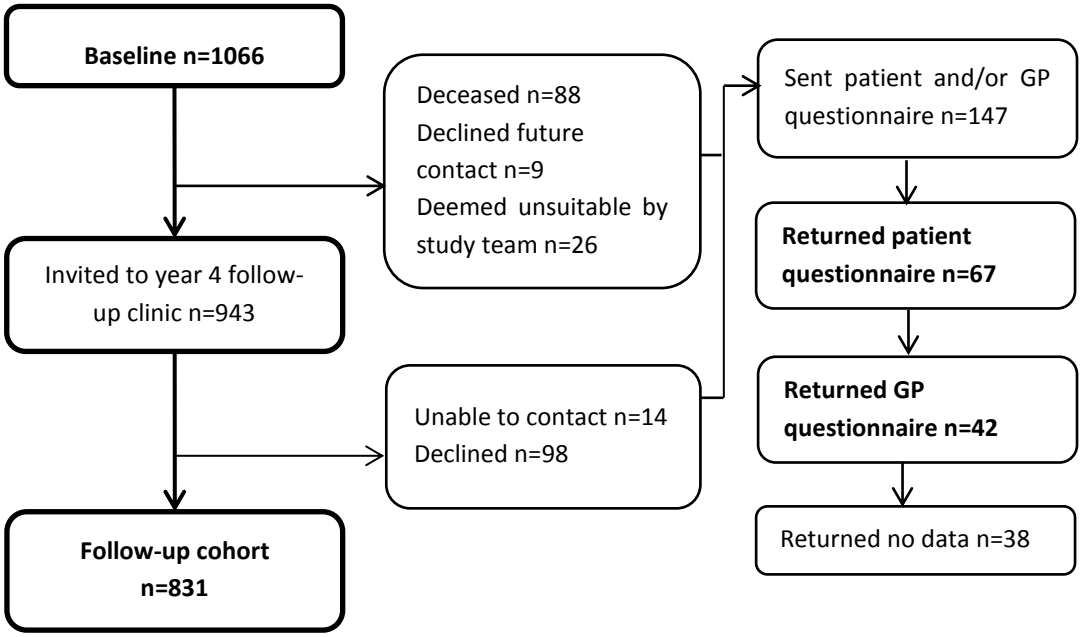


Figure 5-2 Edinburgh Type 2 Diabetes Study follow-up study participation flowchart



Participants in the ET2DS (n=1066) were found to be similar in age and socioeconomic-status distribution compared with those who did not participate. The proportion of men was higher in the recruited cohort (51% vs 42%, $p<0.001$), which reflected the sampling method of the ET2DS which required similar numbers of men and women in each age range. With regards to diabetes history, the two groups had similar durations of diabetes, use of insulin therapy (17% vs 16%, $p>0.05$) and mean plasma HbA1c levels. Two small but statistically significant differences were found between ET2DS participants and non-responders: the ET2DS cohort had a lower mean sBP (133.3mmHg, sd 16.4 vs 137.2mmHg, sd 18.2, $p<0.001$) and a higher mean total cholesterol (4.3mmol/L, sd 0.9 vs 4.2mmol/L, sd 1.0, $p<0.01$).

There were some differences between those subjects that attended the liver sub-study and those that did not - attenders were more likely to be male, have a shorter duration of diabetes and not to use insulin, but these differences were generally quite small and did not reach statistical significance.

Statistically significant differences at follow-up study found attenders were slightly younger, had a lower BP and were of a higher SES. In addition, non-significant differences in attenders were again being male, having a shorter duration of diabetes and not use insulin.

5.1.2 Missing data

The amount of missing data was assessed for each phase of the study (Table 5-2), in subjects attending each of the relevant research clinics.

There was very little missing data at baseline and the liver sub-study (<4.0%) with the exception of CK18 (8.4%), ELF (27.3%) and HA (25.8%) at the liver sub-study. All three variables were measured on stored samples and data were missing due to inadequate sample volumes. In the case of HA, data were already available from baseline, but large amounts of CK18 and ELF missing data were addressed further by imputation, especially as these were key variables for future analysis.

Data imputation was performed using multiple imputation using chained equations as data were considered to be MCAR. This was felt to be appropriate since data were missing due to technical problems or insufficient stored samples and, compared with subjects in whom it was missing, the populations with ELF and CK18 data available had similar clinical and metabolic characteristics (see Table 5-3). Although subjects with CK18 data available were significantly more likely to be male than those in whom it was missing (54% vs 33%), there was no sensible explanation for this finding other than for it to have occurred by chance. The underlying premise for the potential for successful imputation was the correlation between baseline HA and ELF measured at the liver sub-study ($r=0.594$, $p<0.001$). Initial analyses (sections 5.1 to 5.6 and 5.8) were done on the original (un-imputed dataset), following which a sensitivity analysis was run on the imputed dataset (section 5.7).

CK18 data were missing due to random insufficient stored samples. As expected subjects with and without CK18 measures at follow-up were similar for the variables assessed (Table 5.4). LSM measures were missing due to a) inability to obtain readings (105, 70.9%), b) LSM contraindicated (16, 10.9%), and c) missing no reason (27, 18.2%). Subjects who had LSM missing were more likely to be female (63 vs 45%, $p<0.001$), be treated with insulin (30 vs 20%, $p=0.007$), have a larger body habitus (mean BMI 35 vs 31 kg/m2, $p<0.001$, mean waist circumference 111 vs 104cm, $p<0.001$), were less likely to have hepatic steatosis (41 vs 52%, $p=0.020$), and had higher non-specific markers of liver injury (mean AST 32 vs 30 U/L, $p=0.024$, median GGT 20 vs 16 U/L, $p=0.001$)(Table 5-4). Given that this measure was not missing completely at random, and LSM was a key variable required in the follow-up analysis dataset, no imputation was undertaken for follow-up data.

Table 5-1 Baseline characteristics of Edinburgh Type 2 Diabetes Study subjects attending each phase of the study compared with non-responders at baseline and non-attenders at the liver sub-study and follow-up clinics. Values are mean (sd) or % (n).

	ET2DS			*Liver sub-study*			*Follow-up*		
	Participants n=1066	**Non-responders n=4386[a]**	*P[b]*	**Attenders n=939**	**Non-attenders n=127**	*p*	**Attenders n=831**	**Non-attenders n=235**	*p*
Age, years	67.9 (4.20)	67.9 (4.35)	-	67.9 (4.2)	67.8 (4.3)	*0.857*	67.7 (4.2)	68.7 (4.3)	***0.001***
Sex, % male	51.3 (547)	41.9 (1839)	***<0.001***	52.0 (488)	46.5 (59)	*0.257*	51.7 (430)	49.8 (117)	*0.606*
Duration of diabetes, % ≥5 years	51.6 (550)	51.3 (2251)	-	63.1 (587)	66.4 (81)	*0.486*	62.9 (518)	65.5 (150)	*0.486*
HbA1c, %	7.4 (1.1)	7.4 (1.4)	-	7.4 (1.1)	7.5 (1.1)	*0.136*	7.4 (1.1)	7.4 (1.1)	*0.872*
Insulin therapy, %	17.4 (185)	16.1 (704)	-	16.5 (151)	21.3 (27)	*0.146*	16.9 (137)	18.9 (41)	*0.299*
Systolic BP, mmHg	133.3 (16.4)	137.2 (18.2)	***<0.001***	133.2 (16.4)	133.8 (16.8)	*0.712*	132.5 (15.9)	136.1 (18.1)	***0.006***
Total cholesterol, mmol/L	4.3 (0.9)	4.2 (1.0)	***<0.001***	4.3 (0.9)	4.3 (1.0)	*1.000*	4.3 (0.9)	4.2 (0.9)	*0.100*
SIMD quintile, %									
1	11.9 (127)	16.8 (736)		11.6 (109)	14.2 (18)		11.9 (99)	11.9 (28)	
2	19.5 (208)	25.9 (1134)		18.8 (177)	24.4 (31)		17.2 (143)	27.7 (65)	
3	17.6 (188)	18.8 (820)	-	17.1 (161)	21.3 (27)	*0.054*	17.2 (143)	19.1 (45)	***<0.001***
4	18.2 (194)	17.9 (782)		18.1 (170)	18.9 (24)		17.6 (146)	20.4 (48)	
5	32.7 (349)	20.5 (897)		34.3 (322)	21.3 (27)		36.1 (300)	20.9 (49)	

[a] Two invitees had data missing on the Lothian Diabetes Register; [b] p values >0.05 unless stated otherwise (provided by R Marioni)

BP blood pressure; **HbA1c** glycosylated haemoglobin; **SIMD** Scottish Index of Multiple Deprivation.

5.2 Data distributions

This section focuses on the statistical distributions of continuous variables prior to their use in subsequent analyses. Wherever possible variables measured at the liver sub-study were used in subsequent analyses. For a minority of variables, which had only been measured at baseline, baseline levels were used (BMI, CRP, IL6, platelets, TNFα, and waist circumference). It was assumed that these variables were unlikely to alter greatly over the period of one year and were therefore unlikely to affect the results of analyses. The statistical distribution of each variable was assessed and checked for normality ahead of analyses. Distribution histograms are presented in Appendix J and a summary of the findings (mean (sd) or median (IQR)) are show in Table 5-5. Continuous variables collected at the follow-up clinic and used in analyses had the same distributions as those at baseline/liver sub-study.

Table 5-2 Missing data in the Edinburgh Type 2 Diabetes Study at baseline and the liver study. Values are % (n) missing.

	Baseline n=1066	*Liver sub-study n=939*	*Follow-up n=831*
Demographics			
Age	0	0	0
Sex	0	0	0
SIMD	0	0	0
Diabetes history			
Duration of diabetes	1.2% (13)	0.9% (8)	0.8% (7)
Fasting glucose	1.5% (17)	1.6% (15)	1.6% (14)
HbA1c	3.6% (38)	1.3% (12)	1.9% (16)
Treatment type	0.1% (1)	0	0.5% (4)
Cardio-metabolic variables			
BMI	0.1% (1)	-	1.4% (12)
Diastolic BP	0..2% (2)	0.6% (6)	0.4% (3)
eGFR	1.2% (13)	-	-
HDL-cholesterol	0.8% (9)	1.3% (12)	1.4% (12)
Systolic BP	0.2% (2)	0.6% (6)	0.4% (3)
Takes BP lowering meds	0.7% (7)	0	-
Takes lipid lowering meds	0.2% (2)	0	-
Total cholesterol	0.8% (9)	1.3% (12)	1.4% (12)
Triglycerides	-	1.3% (12)	1.4% (12)
Waist circumference	0.5% (5)	-	1.0% (8)
Lifestyle risk factors			
Alcohol excess	1.4% (15)	0.2% (2)	0
Smoking	0	-	0
Liver markers			
ALT	0.8% (9)	0.3% (3)	1.8% (15)
APRI	2.9% (31)	2.7% (25)	2.9% (24)
AST	1.1% (12)	0.7% (7)	1.4% (12)
AST/ALT ratio	1.1% (12)	0.7% (7)	1.8% (15)
CK18	-	8.4% (79)	29.4 (244)
ELF	-	27.3% (256)	3.1% (26)
FIB4	2.9% (31)	2.7% (25)	4.2% (35)
GGT	1.0% (11)	0.6% (6)	2.0% (17)
HA	0.8% (9)	25.8% (242)	3.1% (26)
LSM	-	-	17.8% (148)

	Baseline n=1066	*Liver sub-study n=939*	*Follow-up n=831*
NFS	3.0% (32)	3.1% (29)	5.2% (43)
Platelets	2.0% (21)	-	2.5% (21)
Spleen size	-	0.3% (3)	1.2% (10)
Steatosis (USS)	-	0	1.2% (10)
USS cirrhosis	-	0	1.2% (10)
Other liver related			
Liver screen	-	6.7% (63)	6.3% (52)
Secondary causes for NAFLD	-	0.2% (2)	

ALT alanine aminotransferase; **APRI** aspartate to platelet ratio index; **AST** aspartate aminotransferase; **BMI** body mass index; **BP** blood pressure; **eGFR** estimated glomerular filtration rate; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Score; **GGT** gamma-glutamyl transferase; **HA** hyaluronic acid; **HDL** high density lipoprotein; **NAFLD** non-alcoholic fatty liver disease; **NFS** NAFLD Fibrosis Score; **SIMD** Scottish Index of Multiple Deprivation; **USS** ultrasound scan

Table 5-3 Patient characteristics for variables with high proportions of missing data (liver sub-study visit). Values are mean (sd), median (IQR) or % (n).

	CK18 n=860	No CK18 n=79	*P*	ELF n=683	No ELF n=256	*p*
Age, years	68.9 (4.2)	69.6 (4.0)	*0.142*	68.8 (4.2)	69.3 (4.2)	*0.103*
Sex, % male	53.7 (462)	32.9 (26)	***<0.001***	52.6 (359)	50.4 (129)	*0.558*
Duration of diabetes, % < 5 years	26.2 (223)	22.8 (18)	*0.592*	26.6 (180)	23.9 (61)	*0.450*
HbA1c, %	7.19 (1.0)	7.18 (1.3)	*0.919*	7.23 (1.0)	7.11 (1.1)	*0.131*
Fasting glucose, mmol/l	6.91 (2.3)	6.48 (2.4)	*0.142*	6.92 (2.3)	6.75 (2.2)	*0.310*
Diet controlled, % yes	19.4 (167)	19.0 (15)	*1.000*	19.2 (131)	19.9 (51)	*0.782*
OAHA use, % yes	65.3 (562)	59.5 (47)	*0.325*	66.2 (452)	61.3 (157)	*0.168*
Insulin therapy, % yes	15.2 (131)	21.5 (17)	*0.147*	14.6 (100)	18.8 (48)	*0.132*
BMI, kg/m^2	31.2 (5.6)	32.4 (5.6)	*0.072*	31.2 (5.7)	31.6 (5.6)	*0.298*
Waist circumference, cm	106.6 (12.8)	108.1 (13.5)	*0.343*	106.5 (12.7)	107.5 (13.3)	*0.288*
Total cholesterol, mmol/L	4.2 (0.8)	4.1 (0.8)	*0.538*	4.2 (0.8)	4.1 (0.8)	*0.627*
Systolic BP, mmHg	138.1 (18.1)	138.4 (22.2)	*0.908*	138.5 (17.9)	137.1 (20.0)	*0.291*
Alcohol excess, % yes	13.6 (117)	16.5 (13)	*0.496*	14.4 (98)	12.5 (32)	*0.525*
Ever smoked, % yes	60.2 (518)	62.0 (49)	*0.655*	60.5 (413)	61.1 (156)	*0.882*
Steatosis, % yes	56.6 (487)	60.8 (48)	*0.553*	55.8 (381)	60.2 (154)	*0.237*
ALT, U/L	33.7 (13.1)	31.6 (12.5)	*0.169*	34.0 (13.4)	32.3 (12.3)	*0.081*
AST, U/L	30.6 (10.7)	31.0 (9.2)	*0.786*	30.4 (10.8)	31.3 (9.9)	*0.243*
GGT, U/L	16 (10-29)	19 (10-36)	*0.282*	16 (10-29)	17 (10-31)	*0.316*

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **BMI** body mass index; **BP** blood pressure; **GGT** gamma-glutamyl transferase; **HbA1c** glycosylated haemoglobin; **OAHA** oral anti-hyperglycaemic agent

Table 5-4 Patient characteristics for variables with high proportions of missing data (follow-up visit). Values are mean (sd), median (IQR) or % (n).

	CK18 N=587	No CK18 N=244	*p*	LSM n=683	No LSM n=148	*p*
Age, years	71.4 (4.2)	71.4 (4.1)	*0.920*	71.4 (4.1)	71.2 (4.3)	*0.582*
Sex, % male	52.3 (307)	50.4 (123)	*0.648*	54.9 (375)	37.2 (55)	***<0.001***
Duration of diabetes, % < 5 years	37.3 (217)	36.6 (89)	*0.875*	38.6 (261)	30.6 (45)	*0.074*
HbA1c, %	7.38 (1.2)	7.29 (1.2)	*0.343*	7.31 (1.2)	7.53 (1.3)	*0.069*
Fasting glucose, mmol/l	7.79 (2.9)	7.58 (2.9)	*0.337*	7.68 (2.8)	7.98 (3.2)	*0.298*
Diet controlled, % yes	13.8 (81)	14.0 (34)	*1.000*	14.5 (99)	11.0 (16)	*0.293*
OAHA use, % yes	77.8 (455)	78.5 (190)	*0.854*	78.4 (534)	76.0 (111)	*0.511*
Insulin therapy, % yes	22.7 (133)	18.6 (45)	*0.194*	19.7 (134)	30.1 (44)	***0.007***
BMI, kg/m^2	31.5 (5.8)	31.2 (5.7)	*0.462*	30.7 (5.0)	35.0 (7.4)	***<0.001***
Waist circumference, cm	106.0 (13.0)	104.5 (15.1)	*0.162*	104.4 (12.0)	111.4 (18.4)	***<0.001***
Total cholesterol, mmol/L	4.4 (3.0)	4.2 (0.9)	*0.360*	4.3 (0.9)	4.7 (5.8)	*0.332*
Ever smoked, % yes	59.8 (350)	62.8 (152)	*0.435*	61.1 (416)	58.9 (86)	*0.641*
Steatosis, % yes	52.2 (302)	46.5 (113)	*0.146*	52.4 (358)	41.3 (57)	***0.020***
ALT, U/L	36.1 (12.4)	37.0 (12.8)	*0.365*	36.2 (12.0)	37.3 (14.6)	*0.392*
AST, U/L	30.5 (11.3)	28.8 (9.8)	*0.044*	29.5 (10.0)	32.4 (14.2)	***0.024***
GGT, U/L	17 (10-30)	15 (9-28)	*0.150*	16 (9-27)	20 (12-51)	***0.001***

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **BMI** body mass index; **BP** blood pressure; **GGT** gamma-glutamyl transferase; **HbA1c** glycosylated haemoglobin; **OAHA** oral anti-hyperglycaemic agent

Table 5-5 Summary data for distributions of continuous variables

	Mean (sd), median (IQR) or %(n)	Min.	Max.	Distribution
Diabetes duration, years	7.0 (4.0-12.0)	1.0	44.0	Negative skew
Fasting glucose, mmol/L	6.87 (2.31)	2.0	20.6	Approx. normal
HbA1c, %	7.19 (1.07)	4.3	12.0	Approx. normal
BMI, kg/m^2	31.3 (5.7)	18.4	55.4	Approx. normal
Diastolic BP, mmHg	73.9 (9.6)	34.0	115.0	Approx. normal
eGFR	64.2 (14.6)	8.0	90.0	Approx. normal
HDL-cholesterol	1.23 (0.34)	0.42	2.85	Approx. normal
Systolic BP, mmHg	138.5 (18.3)	89.0	250.0	Approx. normal
Total cholesterol	4.15 (0.80)	2.20	7.70	Approx. normal
Triglycerides	1.66 (0.90)	0.13	9.10	Approx. normal
Waist circumference, cm	106.8 (12.7)	73.0	159.0	Approx. normal
Alcohol, units/week	1.3 (0.0-10.1)	0.0	90.0	Negative skew
AST, U/L	30.7 (10.6)	13.0	94.0	Approx. normal
ALT, U/L	33.6 (13.1)	3.0	135	Approx. normal
GGT, U/L	16.0 (10.0-30.0)	4.0	521.0	Negative skew
CK18, U/L	104.6 (77.7-140.2)	24.4	1000.0	Negative skew
APRI	0.25 (0.20-0.34)	0.07	2.56	Negative skew
AST:ALT ratio	0.96 (0.31)	0.39	6.67	Approx. normal
ELF	8.96 (0.86)	6.89	17.40	Approx. normal
FIB4	1.60 (0.71)	0.47	10.81	Approx. normal
HA,	52.0 (35.74-85.1)	7.7	580.3	Negative skew
NFS	-26.4 (2.4)	-35.2	-16.9	Approx. normal
Platelet count, x10^9	257.7 (68.9)	52.0	606.0	Approx. normal
Spleen size	10.3 (1.6)	6.1	21.3	Approx. normal

ALT alanine aminotransferase; **APRI** aspartate to platelet ratio index; **AST** aspartate aminotransferase; **BMI** body mass index; **BP** blood pressure; **eGFR** estimated glomerular filtration rate; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Score; **GGT** gamma-glutamyl transferase; **HA** hyaluronic acid; **HbA1c** glycosylated haemoglobin; **NAFLD** non-alcoholic fatty liver disease; **NFS** NAFLD Fibrosis Score.

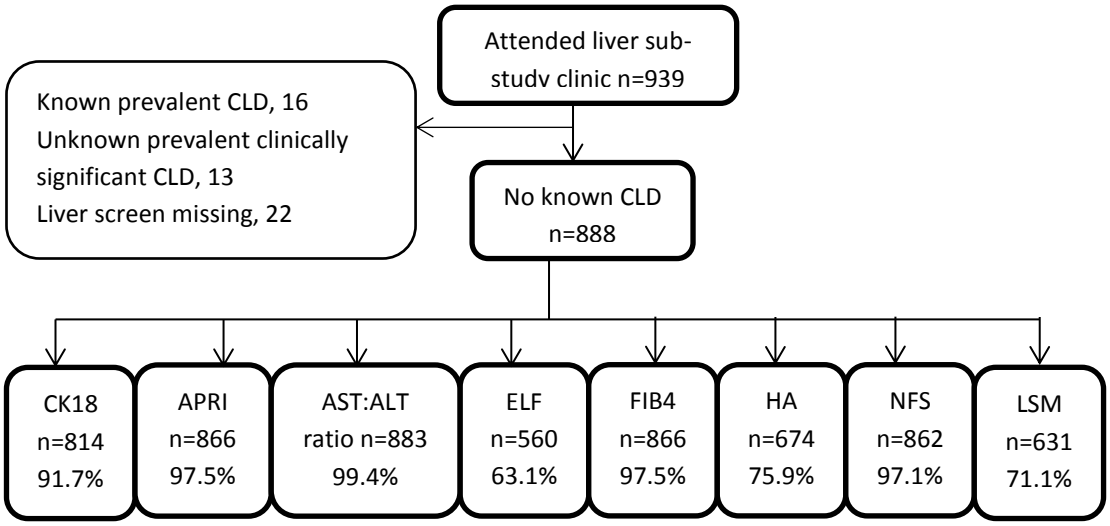
5.3 Difference in levels of steatohepatitis and liver fibrosis markers by gender, steatosis and non-alcoholic fatty liver /disease

Of the 939 ET2DS subjects who underwent liver USS at the liver sub-study research clinic and further physical and liver assessment, 888 did not have pre-diagnosed liver disease or clinically significant CLD on screening (see Figure 5-3). These subjects were considered for inclusion in the current analysis (sections 5.3 to 5.7) which aimed to determine the distribution of steatohepatitis and liver fibrosis markers by gender, steatosis and NAFL/D, as well as factors associated with altered levels of the markers, in a population free of clinical CLD. Details of the data available on the final analysis population are presented in Figure 5-3, and the characteristics of this population are given in Table 5-6.

The presence of steatosis was defined as definite steatosis on USS. NAFL/D was defined as the presence of steatosis on USS in the absence of an alternative cause (i.e. no known CLD, alcohol excess, hepatotoxic medication use or strongly positive autoantibodies. Of the 888 patients included in these analyses 499 (56.2%) had steatosis and 413 (46.5%) had NAFL/D. In determining NAFL/D, of those participants with steatosis, 65 (13.0%) had alcohol excess, 21 (4.2%) hepatotoxic medication use and 2 (0.4%) strongly positive autoantibodies (with 2 participants having more than one of these).

Difference in levels of markers of steatohepatitis and liver fibrosis) according to gender, steatosis and NAFL/D are shown in Table 5-6.

Figure 5-3 Study population and markers available



ALT alanine aminotransferase; **APRI** aspartate to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **CLD** chronic liver disease; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; follow-up follow-up; **HA** hyaluronic acid; liver sub-study liver study; **NFS** NAFLD Fibrosis Score; **LSM** liver stiffness measurement.

Table 5-6 Distribution of markers of steatohepatitis and liver fibrosis by sex, presence of hepatic steatosis and presence of non-alcoholic fatty liver /disease. Values are mean (sd) or median (IQR).

	All			Hepatic steatosis			NAFL/D		
	Male	**Female**	***p***	**Steatosis**	**No steatosis**	***p***	**NAFL/D**	**No NAFL/D**	***p***
CK18	103.0 (80.4-133.6)	99.6 (76.0-136.4)	*0.449*	118.2 (88.6-148.6)	87.3 (68.5-112.0)	***<0.001***	115.3 (87.3-147.3)	92.3 (71.1-121.7)	***<0.001***
APRI	0.27 (0.21-0.35)	0.23 (0.18-0.31)	***<0.001***	0.25 (0.20-0.34)	0.24 (0.19-0.32)	***0.035***	0.25 (0.19-0.33)	0.25 (0.20-0.33)	*0.735*
AST:ALT	0.92 (0.24)	0.98 (0.22)	***<0.001***	0.92 (0.22)	0.99 (0.24)	***<0.001***	0.91 (0.20)	0.98 (0.25)	*<0.001*
ELF	8.82 (0.71)	8.94 (0.74)	*0.053*	8.85 (0.69)	8.92 (0.77)	*0.306*	8.89 (0.69)	8.87 (0.76)	*0.380*
FIB4	1.58 (0.57)	1.43 (0.55)	***<0.001***	1.49 (0.57)	1.53 (0.56)	*0.258*	1.45 (0.55)	1.56 (0.58)	*0.053*
HA	48.7 (34.4-82.2)	52.0 (35.4-82.3)	*0.323*	48.8 (33.6-80.3)	52.3 (35.6-85.1)	***<0.001***	49.9 (35.4-82.9)	50.8 (34.7-81.4)	*0.216*
NFS	-0.30 (1.02)	-0.48 (1.13)	***0.018***	-0.39 (1.11)	-0.37 (1.04)	*0.785*	-0.42 (1.12)	-0.36 (1.03)	***<0.001***
LSM	5.12 (2.56)	5.01 (1.86)	*0.536*	5.46 (2.63)	4.50 (1.44)	***<0.001***	5.31 (2.34)	4.84 (2.17)	***<0.001***

ALT alanine aminotransferase; **APRI** aspartate to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; **HA** hyaluronic acid; **LSM** liver stiffness measurement; **NAFL/D** non-alcoholic fatty liver /disease; **NFS** NAFLD Fibrosis Score;.

5.3.1 Steatohepatitis marker

CK18 values ranged from 29.4 to 993.1 U/L (median 101.2, IQR 76.7-135.0 U/L).

The distribution of CK18 was similarly distributed between men and women (p=0.449), however levels were significantly higher in both those with hepatic steatosis and in those with NAFL/D (p<0.001) compared to those without. CK18 decreased with age (r=-0.10 p=0.003)

5.3.2 Liver fibrosis markers

In the full cohort, APRI ranged from 0.07-1.25 (median 0.25, IQR 0.19-0.33), AST:ALT ranged from 0.39-2.47 (mean 0.95, sd 0.23), ELF scores ranged from 6.89 to 11.60 (mean 8.88, sd 0.73), FIB4 ranged from 0.41-5.58 (mean 1.51, sd 0.57), HA ranged from 7.7-580.3 microg/L (median 50.5 microg/L, IQR 35.0-82.3), NFS ranged from -5.45-4.61 (mean -0.39, sd 1.08) and LSM ranged from 0.37-33.30 kPa (mean 5.07 kPa, sd 2.27).

Males had on average significantly worse (indicating fibrosis) APRI, AST/ALT ratio, FIB4 and NFS. AST:ALT, ELF, FIB4, HA and NFS all increased with age (r=0.22, r=0.28, r=0.26, r=0.29 and r=0.18 respectively, all p<0.001).

LSM was significantly higher in subjects with steatoisis compared with those without steatosis (means 5.46 vs 4.50 kPa, p<0.001). The difference lessened but persisted with the presence of NAFLD. Conversely, HA and AST:ALT ratio were significantly lower in those with steatosis (means 48.8 vs 52.3 microg/L, and 0.92 vs 0.99 respectively, both p<0.001). The same pattern was seen for AST:ALT ratio in the presence of NAFLD, but not for HA. In addition, in NAFL/D, the NFS was significantly lower compared to those without NAFL/D (means -0.42 vs -0.36, p<0.001). Levels were similar for both groups for the remaining fibrosis markers.

5.4 Association of steatohepatitis and liver fibrosis markers with metabolic risk factors and with established hepatotoxic causes

5.4.1 Steatohepatitis marker

Associations of CK18 with metabolic variables and established hepatotoxic causes (excess alcohol intake, positive immunology titres and hepatotoxic medication use) are shown in Table 5-7) for all subjects (n=825) and for those with steatosis (n=460). Higher CK18 levels were significantly associated with hyperglycaemia, increased body fat (higher BMI and waist circumference) and with higher serum triglyceride levels. Levels were notably lower in patients on TZD therapy and in those solely diet controlled. Only the association with waist circumference remained statistically significant when analyses were restricted to subjects with NAFL/D.

CK18 was significantly higher in subjects reporting excess alcohol intake and in those reporting any established hepatotoxic cause.

5.4.2 Liver fibrosis markers

The associations of the liver fibrosis markers with metabolic risk factors and with established hepatotoxic causes are shown in Table 5-7 for all subjects and for those with steatosis.

AST:ALT ratio (Table 5-8), ELF (Table 5-9), FIB4 (Table 5-10) and LSM (Table 5-11) were associated with aspects of hyperglycaemia, measures of body fat and lipids. The NFS (Table 5-12) was not associated with glycaemia, only body fat and lipids.

ELF and HA (Table 5-13) were significantly associated with the duration of diabetes.

In terms of diabetes therapy, there were few statistically significant associations. For ELF, NFS and HA, TZD use compared to non-use was associated with higher (worse) measures. compared with subjects who were treated with diet alone, mean ELF was significantly higher in subjects using OAHA alone and in those using

insulin. For APRI (Table 5-14), FIB4, NFS when compared to diet alone, mean levels were significantly lower in subjects using OAHA and/or insulin.

The associations of ELF, NFS and LSM with metabolic markers remained largely unchanged when restricted to subjects with steatosis or NAFL/D. There was loss of statistical significance for metabolic markers with APRI, AST:ALT ratio and FIB4.

APRI, FIB4 and LSM were significantly higher in those with established hepatotoxic risk factors. ELF, HA and NFS were significantly lower in subjects with established risk factors for liver dysfunction. A significant difference in AST:ALT ratio was only evident in those patients with hepatic steatosis.

Of note, whilst statistically significant all of the correlations were weak with correlation coefficients between 0.07 and 0.24 (with the exception of the stronger association between NFS and measures of body fat, 0.41-0.51).

Table 5-7 Association of CK18 with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All N=814	*p*	Steatosis N=455	*p*	NAFL/D N=261	*p*
Duration of diabetes[a]		0.00	*0.949*	0.03	*0.460*	0.03	*0.570*
Fasting glucose		0.09	***0.012***	0.11	***0.021***	0.06	*0.249*
HbA1c		0.06	*0.070*	0.07	*0.165*	0.05	*0.353*
Treatment:	**Diet**	100.7 (1.81)	***0.047***	110.7 (2.99)	***0.027***	111.4 (3.34)	*0.156*
	Metformin	110.9 (1.11)	*0.077*	128.2 (1.67)	*0.211*	123.9 (1.73)	*0.369*
	Sulph.	109.9 (1.54)	*0.569*	129.7 (2.59)	*0.409*	120.2 (2.40)	*0.728*
	TZD	101.9 (1.94)	*0.124*	117.5 (3.05)	*0.226*	120.5 (3.25)	*0.821*
	Insulin	112.2 (2.36)	*0.348*	135.2 (4.06)	*0.244*	125.3 (4.01)	*0.693*
BMI		0.12	***0.001***	0.03	*0.561*	0.08	*0.125*
Waist circumference		0.14	***<0.001***	0.08	*0.096*	0.14	***0.009***
Total cholesterol		-0.04	*0.391*	-0.04	*0.516*	-0.06	*0.296*
Triglycerides		0.13	***0.002***	0.05	*0.382*	0.08	*0.185*
Established hepatotoxic cause[b]**:**	**No**	122.7 (0.05)	-	142.6 (0.06)	-	NA	-
	Yes	106.7 (0.02)	***0.005***	121.5 (0.03)	***0.013***		
Alcohol excess[c]**:**	**No**	125.2 (0.05)	-	148.4 (0.07)	-	NA	-
	Yes	105.6 (0.02)	***0.003***	121.5 (0.03)	***0.010***		

[a] Analyses on a log10 scale; [b] Defined as alcohol excess, positive autoantibodies (ASmA titer >1:160 or AMA titer >1:40) or use of hepatotoxic medication (non-topicalglucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

BMI body mass index; **CK18** cytokeratin-18; **NAFL/D** non-alcoholic fatty liver /disease; **OAHA** oral anti-hyperglycaemic agents; **Sulph.** sulphonylurea; **TZD** thizolidinedione

Table 5-8 Association of AST:ALT ratio with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All N=883	*p*	Steatosis N=496	*p*	NAFL/D N=286	*p*
Duration of diabetes[a]		0.06	*0.067*	0.09	***0.044***	0.08	*0.109*
Fasting glucose		-0.14	***<0.001***	-0.11	***0.011***	-0.07	*0.137*
HbA1c		-0.16	***<0.001***	-0.13	***0.004***	-0.12	***0.018***
Diabetes treatment:	**Diet**	0.96 (0.02)	*0.387*	0.92 (0.02)	*0.898*	0.88 (0.03)	*0.167*
	Metformin	0.94 (0.01)	*0.245*	0.92 (0.01)	*0.635*	0.91 (0.01)	*0.460*
	Sulph.	0.91 (0.01)	***0.001***	0.89 (0.02)	***0.018***	0.89 (0.02)	*0.182*
	TZD	1.00 (0.02)	***0.002***	0.96 (0.02)	*0.099*	0.95 (0.02)	*0.069*
	Insulin	0.94 (0.02)	*0.782*	0.96 (0.03)	*0.122*	0.95 (0.03)	*0.119*
BMI		-0.08	***0.021***	0.04	*0.369*	0.09	*0.075*
Waist circumference		-0.13	***<0.001***	0.00	*0.967*	0.02	*0.698*
Total cholesterol		0.11	***0.007***	0.11	***0.040***	0.08	*0.189*
Triglycerides		-0.05	*0.233*	0.01	*0.906*	0.03	*0.571*
Established hepatotoxic cause[b]**:**	**No**	0.94 (0.01)	-	0.91 (0.01)	-	NA	-
	Yes	0.97 (0.02)	*0.244*	0.97 (0.02)	***0.023***		
Alcohol excess[c]**:**	**No**	0.94 (0.01)	-	0.91 (0.01)	-	NA	-
	Yes	0.97 (0.02)	*0.273*	0.97 (0.02)	***0.003***		

[a] Analyses on a log10 scale; [b] Defined as alcohol excess, positive autoantibodies (ASmA titer >1:160 or AMA titer >1:40) or use of hepatotoxic medication (non-topical glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **BMI** body mass index; **NAFL/D** non-alcoholic fatty liver /disease; **OAHA** oral anti-hyperglycaemic agents; **Sulph.** sulphonylurea; **TZD** thizolidinedione

Table 5-9 Association of ELF with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All N=560	*p*	Steatosis N=315	*p*	NAFL/D N=176	*p*
Duration of diabetes[a]		0.13	***0.002***	0.19	***0.001***	0.17	***0.006***
Fasting glucose		0.01	*0.860*	0.00	*0.965*	-0.03	*0.682*
HbA1c		0.05	*0.260*	0.01	*0.799*	0.04	*0.519*
Diabetes treatment:	**Diet**	8.71 (0.07)	***0.002***	8.70 (0.10)	***0.042***	8.75 (0.11)	*0.111*
	Metformin	8.93 (0.04)	*0.335*	8.91 (0.05)	*0.205*	8.94 (0.05)	*0.500*
	Sulph.	8.91 (0.06)	*0.888*	8.83 (0.07)	*0.389*	8.86 (0.08)	*0.369*
	TZD	9.12 (0.07)	***0.001***	9.09 (0.09)	***0.012***	9.13 (0.10)	***0.018***
	Insulin	9.17 (0.08)	***<0.001***	9.22 (0.10)	***<0.001***	9.24 (0.11)	***0.002***
BMI		0.11	***0.011***	0.17	***0.003***	0.18	***0.004***
Waist circumference		0.08	***0.075***	0.12	***0.034***	0.15	***0.015***
Total cholesterol		0.01	*0.783*	-0.03	*0.675*	-0.03	*0.722*
Triglycerides		-0.06	*0.228*	-0.11	*0.103*	-0.12	*0.137*
Established hepatotoxic cause[b]**:**	**No**	8.90 (0.03)	-	8.89 (0.04)	-	NA	-
	Yes	8.71 (0.08)	***0.025***	8.69 (0.09)	***0.046***		
Alcohol excess[c]**:**	**No**	8.91 (0.03)	-	8.89 (0.04)	-	NA	-
	Yes	8.66 (0.02)	***0.007***	8.64 (0.10)	***0.026***		

[a] Analyses on a log10 scale; [b] Defined as alcohol excess, positive autoantibodies (ASmA titer >1:160 or AMA titer >1:40) or use of hepatotoxic medication (non-topical glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

BMI body mass index; **ELF** Enhanced Liver Fibrosis panel; **NAFL/D** non-alcoholic fatty liver /disease; **OAHA** oral anti-hyperglycaemic agents; **Sulph.** sulphonylurea; **TZD** thizolidinedione

Table 5-10 Association of FIB4 with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All N=866	*p*	Steatosis N=484	*p*	NAFL/D N=282	*p*
Duration of diabetes[a]		-0.03	*0.323*	0.00	*0.968*	-0.05	*0.308*
Fasting glucose		0.04	*0.297*	0.02	*0.622*	-0.01	*0.772*
HbA1c		-0.11	***0.001***	-0.10	***0.033***	-0.10	***0.042***
Diabetes treatment:	**Diet**	1.71 (0.05)	***<0.001***	1.70 (0.07)	***0.012***	1.50 (0.07)	*0.652*
	Metformin	1.47 (0.03)	***<0.001***	1.47 (0.04)	***0.004***	1.45 (0.03)	*0.191*
	Sulph.	1.43 (0.04)	***0.002***	1.43 (0.06)	***0.033***	1.39 (0.05)	*0.079*
	TZD	1.47 (0.05)	*0.205*	1.48 (0.07)	*0.477*	1.45 (0.07)	*0.739*
	Insulin	1.53 (0.06)	*0.960*	1.53 (0.08)	*0.975*	1.47 (0.08)	*0.939*
BMI		-0.07	***0.047***	0.01	*0.774*	0.05	*0.290*
Waist circumference		-0.04	*0.266*	0.06	*0.164*	0.08	*0.106*
Total cholesterol		-0.05	*0.252*	-0.07	*0.210*	-0.12	***0.047***
Triglycerides		-0.03	*0.527*	0.02	*0.690*	0.04	*0.541*
Established hepatotoxic cause[b]**:**	**No**	1.50 (0.02)	-	1.45 (0.03)	-	NA	-
	Yes	1.63 (0.05)	***0.014***	1.67 (0.06)	***0.002***	NA	-
Alcohol excess[c]**:**	**No**	1.48 (0.02)	-	1.45 (0.03)	-	NA	-
	Yes	1.70 (0.06)	***<0.001***	1.76 (0.07)	***<0.001***	NA	-

[a] Analyses on a log10 scale; [b] Defined as alcohol excess, positive autoantibodies (ASmA titer >1:160 or AMA titer >1:40) or use of hepatotoxic medication (non-topical glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

BMI body mass index; **FIB4** Fibrosis-4 score; **NAFL/D** non-alcoholic fatty liver /disease; OAHA oral anti-hyperglycaemic agents; Sulph. sulphonylurea; TZD thizolidinedione

Table 5-11 Association of LSM with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All N=631	*p*	Steatosis N=371	*p*	NAFL/D N=186	*p*
Duration of diabetes[a]		0.02	*0.608*	0.07	*0.197*	0.08	*0.186*
Fasting glucose		0.09	***0.024***	0.07	*0.160*	0.08	*0.190*
HbA1c		0.12	***0.002***	0.11	***0.040***	0.13	***0.025***
Diabetes treatment:	**Diet**	5.01 (0.20)	*0.668*	5.46 (0.32)	*0.841*	5.21 (0.32)	*0.653*
	Metformin	5.18 (0.12)	*0.251*	5.54 (0.17)	*0.786*	5.39 (0.16)	*0.614*
	Sulph.	5.03 (0.18)	*0.666*	5.27 (0.26)	*0.245*	5.02 (0.26)	*0.135*
	TZD	5.15 (0.23)	*0.794*	5.34 (0.33)	*0.546*	5.42 (0.30)	*0.782*
	Insulin	5.05 (0.24)	*0.862*	5.68 (0.38)	*0.653*	5.63 (0.37)	*0.396*
BMI		0.24	***<0.001***	0.17	***0.001***	0.22	***<0.001***
Waist circumference		0.23	***<0.001***	0.15	***0.004***	0.16	***0.005***
Total cholesterol		-0.08	*0.106*	-0.11	*0.065*	-0.14	***0.032***
Triglycerides		0.06	*0.204*	-0.02	*0.695*	-0.03	*0.675*
Established hepatotoxic cause[b]**:**	**No**	5.01 (0.10)	-	5.31 (0.14)	-	NA	-
	Yes	5.62 (0.23)	***0.016***	6.18 (0.32)	***0.015***	NA	-
Alcohol excess[c]**:**	**No**	4.97 (0.10)	-	5.32 (0.15)	-	NA	-
	Yes	5.77 (0.25)	***0.003***	6.42 (0.37)	***0.005***	NA	-

[a] Analyses on a log10 scale; [b] Defined as alcohol excess, positive autoantibodies (ASmA titer >1:160 or AMA titer >1:40) or use of hepatotoxic medication (non-topical glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

BMI bodymass index; **LSM** liver stiffness measurement; **NAFL/D** non-alcoholic fatty liver /disease; **OAHA** oral anti-hyperglycaemic agent; **Sulph.** sulphonylurea; **TZD** thizolidinedione

Table 5-12 Association of NFS with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All N=862	*p*	Steatosis N=481	*p*	NAFL/D N=282	*p*
Duration of diabetes[a]		0.05	*0.182*	-0.01	*0.756*	-0.06	*0.216*
Fasting glucose		0.05	*0.170*	0.05	*0.283*	0.02	*0.622*
HbA1c		-0.05	*0.181*	-0.03	*0.471*	-0.02	*0.749*
Diabetes treatment:	**Diet**	-0.25 (0.09)	*0.086*	-0.26 (0.12)	*0.310*	-0.36 (0.14)	*0.737*
	Metformin	-0.44 (0.05)	***0.035***	-0.42 (0.06)	*0.079*	-0.44 (0.07)	*0.234*
	Sulph.	-0.52 (0.07)	***0.016***	-0.51 (0.09)	*0.050*	-0.55 (0.10)	*0.090*
	TZD	-0.24 (0.09)	*0.071*	-0.21 (0.12)	*0.135*	-0.26 (0.13)	*0.227*
	Insulin	-0.33 (0.09)	*0.593*	-0.45 (0.14)	*0.428*	-0.45 (0.15)	*0.703*
BMI		0.44	***<0.001***	0.49	***<0.001***	0.51	***<0.001***
Waist circumference		0.41	***<0.001***	0.47	***<0.001***	0.48	***<0.001***
Cholesterol		-0.15	***<0.001***	-0.20	***<0.001***	-0.21	***<0.001***
Triglycerides		0.00	*0.953*	0.03	*0.578*	0.03	*0.553*
Established hepatotoxic cause[b]**:**	**No**	-0.40 (0.04)	-	-0.42 (0.06)	-	NA	-
	Yes	-0.29 (0.09)	***0.031***	-0.29 (0.12)	***0.034***	NA	-
Alcohol excess[c]**:**	**No**	-0.41 (0.04)	-	-0.43 (0.05)	-	NA	-
	Yes	-0.18 (0.11)	***0.042***	-0.17 (0.07)	***0.007***	NA	-

[a] Analyses on a log10 scale; [b] Defined as alcohol excess, positive autoantibodies (ASmA titer >1:160 or AMA titer >1:40) or use of hepatotoxic medication (non-topical glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

BMI body mass index**; NFS** NAFLD Fibrosis Score; **NAFL/D** non-alcoholic fatty liver /disease; **OAHA** oral anti-hyperglycaemic agent; **Sulph.** sulphonylurea; **TZD** thizolidinedione

Table 5-13 Association of HA with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All N=674	*p*	Hepatic steatosis N=374	*p*	NAFL/D N=219	*p*
Duration of diabetes[a]		0.09	***0.027***	0.13	***0.014***	0.13	***0.030***
Fasting glucose		-0.05	*0.218*	-0.09	*0.094*	-0.09	*0.107*
HbA1c		0.02	*0.572*	-0.01	*0.910*	0.00	*0.951*
Diabetes treatment:	**Diet**	49.0 (1.22)	***0.023***	48.0 (1.78)	*0.122*	48.9 (2.05)	*0.187*
	Metformin	55.0 (0.77)	*0.086*	56.6 (0.96)	***0.037***	57.1 (1.14)	*0.121*
	Sulph.	55.5 (1.05)	*0.892*	51.8 (1.35)	*0.362*	52.4 (1.62)	*0.386*
	TZD	70.8 (1.84)	***<0.001***	71.1 (2.35)	***<0.001***	71.3 (2.64)	***0.001***
	Insulin	63.7 (1.85)	***0.023***	65.9 (2.57)	***0.018***	67.0 (2.81)	***0.028***
BMI		0.03	*0.450*	0.11	*0.039*	0.11	*0.057*
Waist circumference		0.02	*0.689*	0.08	*0.102*	0.09	*0.121*
Total cholesterol		-0.03	*0.469*	-0.05	*0.396*	-0.04	*0.584*
Triglycerides		-0.10	***0.028***	-0.11	*0.070*	-0.09	*0.183*
Established hepatotoxic cause[b]**:**	**No**	54.6 (0.03)	-	53.5 (0.04)	-	NA	-
	Yes	47.9 (0.06)	*0.065*	48.4 (0.07)	***0.022***	NA	-
Alcohol excess[c]**:**	**No**	55.1 (0.03)	-	53.5 (0.04)	-	NA	-
	Yes	46.5 (0.07)	***0.021***	46.5 (0.09)	***0.015***	NA	-

[a] Analyses on a log10 scale; [b] Defined as alcohol excess, positive autoantibodies (ASmA titer >1:160 or AMA titer >1:40) or use of hepatotoxic medication (non-topical glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

BMI body mass index**; NFS** NAFLD Fibrosis Score; **NAFL/D** non-alcoholic fatty liver /disease; **OAHA** oral anti-hyperglycaemic agent; **Sulph.** sulphonylurea; **TZD** thizolidinedione

Table 5-14 Association of APRI with metabolic and established hepatic risk factors. Values are age and sex adjusted correlation coefficients or mean (SEM)

		All N=866	*p*	Steatosis N=484	*p*	NAFL/D N=282	*p*
Duration of diabetes[a]		-0.07	***0.039***	-0.06	*0.201*	-0.10	*0.056*
Fasting glucose		0.07	*0.051*	0.04	*0.328*	-0.02	*0.661*
HbA1c		-0.06	*0.066*	-0.08	*0.067*	-0.10	***0.049***
Treatment:	**Diet**	0.23 (0.01)	***<0.001***	0.30 (0.01)	***0.006***	0.28 (0.01)	*0.083*
	Metformin	0.54 (0.01)	***<0.001***	0.26 (0.01)	***0.001***	0.25 (0.01)	***0.022***
	Sulph.	0.24 (0.01)	*0.060*	0.26 (0.01)	*0.246*	0.25 (0.01)	*0.247*
	TZD	0.23 (0.01)	***0.001***	0.25 (0.01)	***0.039***	0.24 (0.01)	*0.120*
	Insulin	0.26 (0.01)	*0.826*	0.26 (0.01)	*0.704*	0.25 (0.01)	*0.520*
BMI		-0.02	*0.479*	0.02	*0.588*	0.06	*0.206*
Waist circumference		0.01	*0.779*	0.07	*0.109*	0.10	*0.054*
Total cholesterol		-0.06	*0.140*	-0.07	*0.192*	-0.11	*0.058*
Triglycerides		0.02	*0.606*	0.03	*0.582*	0.04	*0.462*
Established hepatotoxic cause[b]**:**	**No**	0.25 (0.02)	-	0.25 (0.02)	-	NA	-
	Yes	0.29 (0.04)	***0.001***	0.31 (0.05)	***<0.001***		
Alcohol excess[c]**:**	**No**	0.25 (0.01)	-	0.25 (0.05)	-	NA	-
	Yes	0.31 (0.04)	***<0.001***	0.33 (0.05)	***<0.001***		

[a] Analyses on a log10 scale; [b] Defined as alcohol excess, positive autoantibodies (ASmA titer >1:160 or AMA titer >1:40) or use of hepatotoxic medication (non-topical glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

APRI aspartate to platelet ratio index; **BMI** body mass index; **NAFL/D** non-alcoholic fatty liver /disease; **OAHA** oral anti-hyperglycaemic agents; **Sulph.** sulphonylurea; **TZD** thizolidinedione

5.5 Characteristics of subjects in highest steatohepatitis and liver fibrosis marker quintiles

Since particularly high levels of CK18 and ELF may be diagnostic of clinically important liver inflammation and fibrosis respectively, we determined the clinical characteristics of subjects in the top marker quintiles (Table 5-15 through Table 5-22).

5.5.1 Steatohepatitis marker

Data for CK18 is shown in Table 5-15

Compared with subjects in the bottom four quintiles, subjects in the highest CK18 quintile were significantly older and had significantly higher indices of hyperglycaemia, higher triglyceride levels and increased body fat. In addition, more were on intensive diabetes treatment (including insulin) and more reported drinking excess alcohol. There was a significantly higer proportion of patients with hepatic steatosis in the highest quintile (49 vs 84%).

When the analyses were restricted to subjects with hepatic steatosis only only measures of poorer diabetes control and alcohol excess remained significant. When analyses were restricted to subjects with NAFL/D, no statistically significant differences were found.

5.5.2 Liver fibrosis markers

Associations between the highest marker quintiles and potential risk factors were varied according to the specific marker. Full data is shown in Table 5-16 through Table 5-22.

In general, subjects in the highest quintiles were older (for AST:ALT ratio, ELF, FIB4, HA and NFS). Only APRI was associated with poorer glucose control. Both HA and NFS had shorter diabetes durations in the top quintile. In general (although

not always reaching statistical significance) there were larger proportions of patients using TZD therapy in the higher marker quintiles compared to the lower four (ELF, HA, NFS and LSM). Measures of body fat were significantly higher for the top quintiles of NFS and LSM. A higher proportion of alcohol excess was associated with APRI, FIB4 and LSM. Conversely, the highest ELF and HA quintiles were associated with a lower alcohol intake.

It should be noted that the highest quintile of AST:ALT ratio was generally associated with the opposite findings to all of the other markers: being female, better diabetes control, lower body fat measures and less hepatic steatosis (Table 5-17).

When subjects were restricted to those with steatosis or NAFL/D the majority of associations lost statistical significance.

Table 5-15 Risk factors in highest versus lower quintiles of CK18. Values are mean (sd) or proportion (n)

		All			Steatosis			NAFL/D		
		Q1-Q4 N=652	Q5 N=162	*p*	Q1-Q4 N=368	Q5 N=92	*p*	Q1-Q4 N=303	Q5 N=75	*p*
Age		69.0 (4.1)	68.1 (4.5)	***0.017***	68.5 (3.9)	68.0 (4.5)	*0.404*	68.5 (4.0)	67.9 (4.3)	*0.221*
Sex, % male		54.6 (356)	52.5 (85)	*0.660*	50.5 (186)	55.4 (51)	*0.417*	48.2 (146)	50.7 (38)	*0.797*
Duration of diabetes, % ≤5years		38.6 (250)	34.8 (55)	*0.419*	40.1 (146)	37.8 (34)	*0.719*	59.8 (180)	60.8 (45)	*0.895*
Diabetes treatment:	**Diet**	22.1 (144)	11.1 (18)	***0.001***	19.3 (71)	8.7 (8)	***0.014***	*17.5 (53)*	12.0 (9)	*0.298*
	Metformin	61.8 (403)	71.0 (115)	***0.036***	70.1 (258)	75.0 (69)	*0.372*	*71.3 (216)*	74.7 (56)	*0.667*
	Sulph.	31.0 (202)	35.2 (57)	*0.302*	30.2 (111)	37.0 (34)	*0.212*	*30.0 (91)*	30.7 (23)	*1.000*
	TZD	19.3 (126)	13.6 (22)	*0.110*	19.3 (71)	15.2 (14)	*0.453*	*19.8 (60)*	21.3 (16)	*0.750*
	Insulin	13.8 (90)	18.5 (30)	*0.138*	12.8 (47)	19.6 (18)	*0.097*	*14.2 (43)*	17.3 (13)	*0.473*
Fasting glucose, mmol/L		6.80 (2.24)	7.33 (2.32)	***0.010***	6.99 (2.3)	7.79 (2.5)	***0.004***	6.98 (2.4)	7.48 (2.1)	*0.104*
HbA1c, %		7.14 (1.03)	7.36 (1.08)	***0.020***	7.26 (1.1)	7.54 (1.1)	***0.027***	7.27 (1.1)	7.47 (1.0)	*0.160*
Body mass index, kg/m^2		31.0 (5.6)	32.1 (5.7)	***0.020***	32.3 (5.5)	32.5 (5.4)	*0.698*	32.3 (5.6)	33.0 (5.6)	*0.303*
Waist circumference, cm		106.1 (12.7)	108.8 (12.6)	***0.016***	108.6 (12.1)	109.9 (11.1)	*0.351*	108.1 (12.0)	110.0 (11.7)	*0.240*
Total cholesterol, mmol/L		4.15 (0.79)	4.16 (0.75)	*0.917*	4.22 (0.8)	4.20 (0.8)	*0.828*	4.23 (0.8)	4.11 (0.8)	*0.364*
Triglycerides[a], mmol/L		1.61 (0.91)	1.92 (1.11)	***0.009***	0.23 (0.2)	0.27 (0.2)	*0.190*	0.23 (0.2)	0.29 (0.2)	*0.083*
Steatosis, % 'fatty'		48.9 (319)	84.0 (136)	***<0.001***	-	-	-	-	-	-
Excess alcohol intake[b]		10.3 (67)	17.9 (29)	***0.010***	11.4 (42)	20.7 (19)	***0.025***	-	-	-
Established hepatotoxic cause[c]		21.3 (139)	24.1 (39)	*0.458*	15.8 (58)	26.1 (24)	***0.032***	-	-	-

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic

CK18 cytokeratin-18; **HbA1c** glycosylated haemoglobin; **Sulph.** sulphonylurea; **TZD** thiozolidinedione

Table 5-16 Risk factors in highest versus lower quintiles of APRI. Values are mean (sd) or proportion (n)

		All			Steatosis			NAFL/D		
		Q1-Q4 N=693	Q5 N=173	*p*	Q1-Q4 N=393	Q5 N=98	*p*	Q1-Q4 N=323	Q5 N=80	*p*
Age, years		68.8 (4.2)	69.1 (4.2)	*0.489*	68.6 (4.0)	68.0 (4.2)	*0.197*	68.5 (4.0)	68.1 (0.5)	*0.373*
Sex, % male		50.2 (348)	59.5 (103)	***0.033***	49.4 (194)	51.0 (50)	*0.822*	45.2 (146)	50.0 (40)	*0.455*
Duration of diabetes, % ≤5years		38.6 (265)	32.6 (56)	*0.159*	39.4 (153)	41.2 (40)	*0.817*	39.7 (127)	42.5 (34)	*0.703*
Diabetes treatment:	**Diet**	17.9 (124)	28.3 (49)	***0.003***	16.5 (65)	22.4 (22)	*0.184*	16.4 (53)	18.8 (15)	*0.619*
	Metformin	65.9 (457)	53.2 (92)	***0.003***	73.0 (287)	61.2 (60)	***0.026***	73.7 (238)	63.8 (51)	*0.096*
	Sulph.	32.2 (233)	28.3 (49)	*0.360*	32.6 (128)	27.6 (27)	*0.395*	30.3 (98)	30.0 (24)	*1.000*
	TZD	19.8 (137)	10.4 (18)	***0.004***	19.3 (76)	13.3 (13)	*0.188*	20.4 (66)	16.3 (13)	*0.436*
	Insulin	14.4 (100)	18.5 (32)	*0.194*	13.0 (51)	19.4 (19)	*0.108*	14.2 (46)	17.5 (14)	*0.484*
Fasting glucose, mmol/L		6.74 (2.2)	7.19 (2.3)	***0.017***	6.95 (2.3)	7.47 (2.4)	*0.051*	6.93 (2.4)	7.12 (2.2)	*0.557*
HbA1c, %		7.16 (1.0)	7.17 (1.0)	*0.888*	7.27 (1.1)	7.36 (1.0)	*0.454*	7.28 (1.1)	7.31 (0.98)	*0.810*
Body mass index, kg/m^2		31.2 (5.5)	31.3 (5.9)	*0.867*	32.2 (5.2)	33.2 (6.4)	*0.103*	32.2 (5.2)	33.7 (6.9)	*0.076*
Waist circumference, cm		106.4 (12.7)	107.6 (13.4)	*0.272*	108.4 (11.4)	111.0 (13.8)	*0.057*	107.8 (11.4)	111.0 (14.2)	***0.034***
Total cholesterol, mmol/L		4.17 (0.8)	4.04 (0.77)	*0.077*	4.23 (0.8)	4.07 (0.8)	*0.119*	4.23 (0.8)	0.40 (0.9)	*0.058*
Triglycerides[a], mmol/L		0.16 (0.2)	0.18 (0.2)	*0.305*	0.23 (0.2)	0.24 (0.2)	*0.575*	0.23 (0.2)	0.27 (0.2)	*0.167*
Steatosis, % 'fatty'		53.8 (373)	64.2 (111)	*0.016*	-	-	-	-	-	-
Excess alcohol intake[b]		10.1 (70)	19.1 (33)	***0.002***	10.9 (43)	24.5 (24)	***0.001***	-	-	-
Established hepatotoxic cause[c]		20.8 (144)	23.7 (41)	*0.408*	15.0 (59)	29.6 (29)	***0.002***	-	-	-

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic

APRI aspartate to platelet ratio index; **HbA1c** glycosylated haemoglobin; **Sulph.** sulphonylurea; **TZD** thiozolidinedione

Table 5-17 Risk factors in highest versus lower quintiles of AST:ALT ratio. Values are mean (sd) or proportion (n)

	All			Steatosis			NAFL/D		
	Q1-Q4 N=706	**Q5 N=177**	***p***	**Q1-Q4 N=402**	**Q5 N=100**	***p***	**Q1-Q4 N=331**	**Q5 N=82**	***p***
Age	68.5 (4.1)	70.3 (4.2)	***<0.001***	68.2 (3.4)	69.6 (4.2)	***0.002***	68.2 (4.0)	69.4 (4.2)	***0.015***
Sex, % male	54.4 (384)	43.5 (77)	***0.011***	52.5 (211)	38.0 (38)	***0.010***	49.8 (165)	30.5 (25)	***0.002***
Duration of diabetes, % ≤5years	37.4 (262)	36.6 (64)	*0.862*	40.2 (159)	37.0 (37)	*0.647*	39.9 (131)	39.0 (32)	*0.900*
Diet	19.5 (138)	20.9 (37)	*0.674*	17.9 (72)	16.0 (16)	*0.769*	17.2 (57)	14.6 (12)	*0.624*
Metformin	64.9 (458)	58.2 (103)	*0.116*	71.9 (289)	67.0 (67)	*0.328*	72.5 (240)	69.5 (57)	*0.585*
Diabetes treatment: Sulph.	33.3 (235)	23.2 (41)	***0.011***	32.1 (129)	28.0 (28)	*0.471*	30.8 (102)	26.8 (22)	*0.505*
TZD	16.7 (118)	22.0 (39)	*0.100*	17.4 (70)	19.0 (19)	*0.770*	19.0 (63)	19.5 (16)	*1.000*
Insulin	15.0 (106)	15.8 (28)	*0.815*	12.9 (52)	20.0 (20)	*0.080*	13.3 (44)	22.0 (18)	*0.058*
Fasting glucose, mmol/L	6.98 (2.3)	6.42 (2.3)	***0.004***	7.19 (2.3)	6.75 (2.5)	*0.096*	7.09 (2.2)	6.81 (2.9)	*0.335*
HbA1c, %	7.25 (1.1)	6.91 (0.9)	***<0.001***	7.36 (1.1)	7.15 (0.9)	*0.055*	7.34 (1.1)	7.21 (1.0)	*0.337*
Body mass index, kg/m^2	31.6 (5.6)	30.1 (5.8)	***0.002***	32.4 (5.3)	32.4 (6.1)	*0.997*	32.4 (5.5)	32.8 (6.2)	*0.573*
Waist circumference, cm	107.6 (12.4)	103.1 (13.9)	***<0.001***	109.0 (11.6)	108.6 (13.3)	*0.755*	108.6 (11.8)	108.1 (13.3)	*0.710*
Total cholesterol, mmol/L	4.12 (0.8)	4.24 (0.7)	*0.098*	4.18 (0.8)	4.28 (0.8)	*0.340*	4.16 (0.8)	4.31 (0.8)	*0.174*
Triglycerides[a], mmol/L	0.18 (0.2)	0.13 (0.2)	***0.033***	0.23 (0.2)	0.24 (0.2)	*0.507*	0.23 (0.2)	0.26 (0.2)	*0.276*
Steatosis, % 'fatty'	60.2 (425)	40.1 (71)	***<0.001***	-	-	-	-	-	-
Excess alcohol intake[b]	11.3 (80)	13.6 (24)	*0.433*	12.2 (49)	19.0 (19)	*0.101*	-	-	-
Established hepatotoxic cause[c]	20.3 (143)	24.9 (44)	*0.182*	15.7 (63)	26.0 (26)	***0.019***	-	-	-

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **HbA1c** glycosylated haemoglobin; **Sulph.** sulphonylurea; **TZD** thiozolidinedione

Table 5-18 Risk factors in highest versus lower quintiles of ELF. Values are mean (sd) or proportion (n)

		All			Steatosis			NAFL/D		
		Q1-Q4 N=448	Q5 N=112	*p*	Q1-Q4 N=255	Q5 N=64	*p*	Q1-Q4 N=207	Q5 N=52	*p*
Age		68.3 (4.1)	70.5 (3.8)	***<0.001***	67.9 (3.9)	69.7 (4.0)	***0.002***	67.8 (3.9)	69.4 (4.0)	***0.017***
Sex, % male		51.3 (230)	46.4 (52)	*0.398*	49.0 (125)	46.9 (30)	*0.781*	43.0 (89)	48.1 (25)	*0.535*
Duration of diabetes, % ≤5years		40.8 (181)	32.7 (36)	*0.128*	41.8 (105)	36.5 (23)	*0.476*	42..0 (86)	33.3 (17)	*0.338*
Diabetes treatment:	**Diet**	20.1 (90)	14.3 (16)	*0.179*	16.9 (43)	14.1 (9)	*0.706*	16.9 (35)	9.6 (5)	*0.282*
	Metformin	65.4 (293)	68.8 (77)	*0.577*	71.8 (183)	73.4 (47)	*0.877*	72.0 (149)	78.8 (41)	*0.382*
	Sulph.	31.3 (140)	32.1 (36)	*0.909*	31.4 (80)	25.0 (16)	*0.363*	29.0 (60)	25.0 (13)	*0.610*
	TZD	17.6 (79)	23.2 (26)	*0.178*	17.6 (45)	25.0 (16)	*0.213*	19.3 (40)	26.9 (14)	*0.253*
	Insulin	12.3 (55)	23.2 (26)	***0.006***	12.5 (32)	26.6 (17)	***0.011***	13.5 (28)	26.9 (14)	***0.033***
Fasting glucose, mmol/L		6.88 (2.2)	6.97 (2.5)	*0.722*	7.13 (2.3)	6.99 (2.7)	*0.697*	7.07 (2.3)	6.87 (2.5)	*0.583*
HbA1c, %		7.19 (1.0)	7.25 (1.1)	*0.596*	7.35 (1.1)	7.23 (1.1)	*0.435*	7.35 (1.1)	7.30 (1.2)	*0.764*
Body mass index, kg/m^2		30.9 (5.5)	31.9 (6.1)	*0.096*	31.9 (5.2)	33.4 (6.5)	*0.040*	32.1 (5.6)	33.5 (6.5)	*0.129*
Waist circumference, cm		105.8 (12.2)	108.1 (13.5)	*0.082*	107.7 (11.4)	110.5 (13.4)	*0.091*	107.2 (11.8)	1110.6 (13.6)	*0.176*
Total cholesterol, mmol/L		4.17 (0.8)	4.13 (0.8)	*0.670*	4.27 (0.8)	4.07 (0.8)	*0.135*	4.28 (0.8)	4.03 (0.7)	*0.094*
Triglycerides[a], mmol/L		0.16 (0.2)	0.19 (0.2)	*0.358*	0.24 (0.2)	0.22 (0.2)	*0.497*	0.25 (0.2)	0.22 (0.2)	*0.357*
Steatosis, % 'fatty'		57.1 (256)	52.7 (59)	*0.397*	-	-	-	-	-	-
Excess alcohol intake[b]		13.2 (59)	9.0 (10)	*0.262*	157 (40)	7.8 (5)	*0.158*	-	-	-
Established hepatotoxic cause[c]		23.7 (106)	19.63 (22)	*0.450*	20.8 (53)	10.9 (7)	*0.076*	-	-	-

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic

ELF Enhanced Liver Fibrosis panel; **HbA1c** glycosylated haemoglobin; **Sulph.** sulphonylurea; **TZD** thiozolidinedione

Table 5-19 Risk factors in highest versus lower quintiles of FIB4. Values are mean (sd) or proportion (n)

		All			Steatosis			NAFL/D		
		Q1-Q4 N=693	Q5 N=173	*p*	Q1-Q4 N=393	Q5 N=98	*p*	Q1-Q4 N=323	Q5 N=80	*p*
Age		68.6 (4.2)	70.0 (3.9)	***<0.001***	68.3 (4.0)	69.2 (4.1)	***0.046***	68.3 (4.1)	69.1 (4.0)	*0.086*
Sex, % male		50.8 (352)	57.2 (99)	*0.148*	49.1 (193)	52.0 (51)	*0.652*	44.9 (145)	51.2 (41)	*0.319*
Duration of diabetes, % ≤5years		38.0 (261)	34.9 (60)	*0.481*	59.0 (229)	64.9 (63)	*0.299*	40.0 (128)	41.3 (33)	*0.899*
Diabetes treatment:	**Diet**	17.7 (123)	28.9 (50)	***0.002***	16.8 (66)	21.4 (21)	*0.301*	17.3 (56)	15.0 (12)	*0.739*
	Metformin	66.4 (460)	51.4 (89)	***<0.001***	72.5 (285)	63.3 (62)	*0.083*	72.8 (235)	67.5 (54)	*0.405*
	Sulph.	32.5 (225)	27.2 (47)	*0.200*	32.8 (129)	26.5 (26)	*0.274*	31.3 (101)	26.3 (21)	*0.417*
	TZD	18.9 (131)	13.9 (24)	*0.149*	19.6 (77)	12.2 (12)	*0.107*	21.4 (69)	12.5 (10)	*0.084*
	Insulin	15.3 (106)	15.0 (26)	*1.000*	13.2 (52)	18.4 (18)	*0.198*	13.9 (45)	18.8 (15)	*0.294*
Fasting glucose, mmol/L		6.77 (2.2)	7.08 (2.4)	*0.102*	6.95 (2.3)	7.47 (2.5)	***0.049***	6.91 (2.3)	7.21 (2.6)	*0.307*
HbA1c, %		7.19 (1.0)	7.06 (1.0)	*0.168*	7.29 (1.1)	7.28 (1.0)	*0.937*	7.28 (1.1)	7.30 (1.0)	*0.873*
Body mass index, kg/m^2		31.4 (5.5)	30.8 (6.2)	*0.214*	32.3 (5.2)	32.8 (6.5)	*0.406*	32.2 (5.2)	33.7 (6.8)	*0.072*
Waist circumference, cm		106.8 (12.5)	105.6 (14.0)	*0.279*	108.4 (11.5)	110.7 (13.4)	*0.087*	107.8 (11.5)	111.3 (13.8)	***0.018***
Total cholesterol, mmol/L		4.16 (0.8)	4.08 (0.8)	*0.285*	4.23 (0.8)	4.08 (0.8)	*0.142*	4.24 (0.8)	4.01 (0.8)	*0.053*
Triglycerides[a], mmol/L		0.17 (0.2)	0.15 (0.2)	*0.218*	0.23 (0.2)	0.23 (0.2)	*0.856*	0.23 (0.2)	0.24 (0.2)	*0.867*
Steatosis, % 'fatty'		57.3 (397)	50.3 (87)	*0.104*	-	-	-	-	-	-
Excess alcohol intake[b]		10.3 (71)	18.6 (32)	***0.004***	10.7 (42)	25.5 (25)	***<0.001***	-	-	-
Established hepatotoxic cause[c]		20.8 (144)	23.7 (41)	*0.408*	15.3 (60)	28.6 (28)	***0.003***	-	-	-

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic

FIB4 Fibrosis-4 Score; **HbA1c** glycosylated haemoglobin; **Sulph.** sulphonylurea; **TZD** thiozolidinedione

Table 5-20 Risk factors in highest versus lower quintiles of HA. Values are mean (sd) or proportion (n)

		All			Steatosis			NAFL/D		
		Q1-Q4 N=540	Q5 N=134	*p*	Q1-Q4 N=304	Q5 N=76	*p*	Q1-Q4 N=245	Q5 N=61	*p*
Age		68.3 (4.0)	70.7 (3.9)	***<0.001***	68.0 (3.9)	69.5 (4.2)	***0.003***	68.0 (3.9)	69.1 (4.1)	*0.052*
Sex, % male		53.0 (286)	54.5 (73)	*0.772*	50.0 (152)	51.3 (39)	*0.898*	45.7 (112)	45.9 (28)	*1.000*
Duration of diabetes, % ≤5years		41.1 (220)	30.3 (40)	***0.022***	41.5 (124)	34.7 (26)	*0.295*	41.6 (101)	33.3 (20)	*0.333*
Diabetes treatment:	**Diet**	20.0 (108)	18.7 (25)	*0.809*	16.4 (50	15.8 (12)	*1.000*	15.5 (38)	16.4 (10)	*0.846*
	Metformin	65.0 (351)	64.2 (86)	*0.920*	72.0 (219)	73.7 (56)	*0.886*	72.7 (178)	75.4 (46)	*0.748*
	Sulph.	31.9 (172)	34.3 (46)	*0.607*	33.2 (101)	26.3 (20)	*0.273*	31.4 (77)	21.3 (13)	*0.157*
	TZD	16.1 (87)	25.4 (34)	***0.017***	16.4 (50)	28.9 (22)	***0.021***	18.0 (44)	29.5 (18)	*0.051*
	Insulin	13.3 (72)	17.2 (23)	*0.268*	13.2 (40)	19.7 (15)	*0.148*	14.3 (35)	21.3 (13)	*0.174*
Fasting glucose, mmol/L		7.02 (2.2)	6.65 (2.4)	*0.095*	7.30 (2.3)	6.58 (2.5)	***0.017***	7.23 (2.3)	6.51 (2.32)	***0.030***
HbA1c, %		7.22 (1.1)	7.22 (1.0)	*0.978*	7.40 (1.1)	7.23 (1.1)	*0.250*	7.40 (1.1)	7.24 (1.1)	*0.308*
Body mass index, kg/m^2		31.2 (5.8)	31.0 (5.2)	*0.646*	32.2 (5.6)	32.6 (5.4)	*0.520*	32.2 (5.3)	32.4 (5.3)	*0.890*
Waist circumference, cm		106.5 (12.8)	106.3 (12.4)	*0.846*	108.5 (12.0)	108.9 (11.7)	*0.755*	108.1 (12.5)	107.5 (10.7)	*0.752*
Total cholesterol, mmol/L		4.18 (0.8)	4.06 (0.8)	*0.185*	4.26 (0.8)	4.09 (0.9)	*0.175*	4.48 (0.8)	4.05 (0.9)	*0.107*
Triglycerides[a], mmol/L		0.17 (0.2)	0.15 (0.2)	*0.372*	0.25 (0.2)	0.21 (0.2)	*0.226*	0.25 (0.2)	0.21 (0.2)	*0.192*
Steatosis, % 'fatty'		57.2 (309)	48.5 (65)	*0.080*	-	-	-	-	-	-
Excess alcohol intake[b]		13.5 (73)	9.8 (13)	*0.310*	15.5 (47)	11.8 (9)	*0.475*	-	-	-
Established hepatotoxic cause[c]		23.5 (127)	19.4 (26)	*0.357*	20.7 (63)	14.5 (11)	*0.259*	-	-	-

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic

HA hyaluronic acid; **HbA1c** glycosylated haemoglobin; **Sulph.** sulphonylurea; **TZD** thiozolidinedione.

Table 5-21 Risk factors in highest versus lower quintiles of NFS. Values are mean (sd) or proportion (n)

		All			Steatosis			NAFL/D		
		Q1-Q4 N=690	Q5 N=172	*p*	Q1-Q4 N=391	Q5 N=97	*p*	Q1-Q4 N=321	Q5 N=80	*p*
Age		68.7 (4.1)	69.6 (4.2)	***0.013***	68.3 (4.0)	68.8 (4.4)	*0.396*	68.4 (4.0)	68.6 (4.4)	*0.611*
Sex, % male		51.4 (355)	54.7 (94)	*0.495*	49.4 (193)	51.5 (50)	*0.734*	46.7 (150)	45.0 (36)	*0.803*
Duration of diabetes, % ≤5years		39.4 (269)	30.2 (52)	***0.028***	40.5 (156)	38.1 (37)	*0.728*	39.9 (127)	42.5 (34)	*0.703*
Diabetes treatment:	**Diet**	19.9 (137)	20.3 (35)	*0.915*	17.9 (70)	16.5 (16)	*0.882*	17.8 (57)	13.8 (11)	*0.505*
	Metformin	64.6 (446)	59.3 (102)	*0.215*	71.9 (281)	67.0 (65)	*0.382*	72.9 (234)	67.5 (54)	*0.335*
	Sulph.	32.6 (225)	26.2 (45)	*0.118*	32.5 (127)	27.8 (27)	*0.396*	30.8 (99)	27.5 (22)	*0.589*
	TZD	17.1 (118)	21.5 (37)	*0.184*	17.9 (70)	19.6 (19)	*0.769*	19.6 (63)	20.0 (16)	*1.000*
	Insulin	14.8 ()102	16.3 (28)	*0.634*	13.6 (53)	15.5 (15)	*0.625*	14.0 (45)	16.3 (14)	*0.597*
Fasting glucose, mmol/L		6.83 (2.2)	6.85 (2.4)	*0.922*	7.05 (2.3)	7.15 (2.6)	*0.708*	7.00 (2.3)	6.88 (2.4)	*0.664*
HbA1c, %		7.18 (1.1)	7.07 (1.0)	*0.198*	7.30 (1.1)	7.23 (1.0)	*0.583*	7.29 (1.1)	7.24 (0.9)	*0.725*
Body mass index, kg/m^2		30.1 (4.8)	36.0 (6.2)	***<0.001***	31.1 (4.5)	37.5 (6.0)	***<0.001***	31.1 (4.6)	37.9 (6.1)	***<0.001***
Waist circumference, cm		104.1 (11.4)	116.8 (13.3)	***<0.001***	106.2 (10.6)	119.5 (11.4)	***<0.001***	105.8 (10.8)	119.0 (11.3)	***<0.001***
Total cholesterol, mmol/L		4.19 (0.8)	3.95 (0.7)	***0.003***	4.25 (0.8)	4.00 (0.7)	***0.026***	4.25 (0.8)	4.0 (0.8)	***0.021***
Triglycerides[a], mmol/L		0.16 (0.2)	0.18 (0.2)	*0.602*	0.23 (0.2)	0.22 (0.2)	*0.822*	0.24 (0.2)	0.22 (0.2)	*0.541*
Steatosis, % 'fatty'		56.2 (388)	54.1 (93)	*0.608*	-	-	-	-	-	-
Excess alcohol intake[b]		11.2 (77)	14.6 (25)	*0.234*	12.5 (49)	18.6 (18)	*0.138*	-	-	-
Established hepatotoxic cause[c]		21.0 (145)	22.1 (38)	*0.755*	16.9 (66)	21.6 (21)	*0.300*	-	-	-

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic

HbA1c glycosylated haemoglobin; **NFS** NAFLD Fibrosis Score; **Sulph.** sulphonylurea; **TZD** thiozolidinedione

Table 5-22 Risk factors in highest versus lower quintiles of LSM. Values are mean (sd) or proportion (n)

		All			Steatosis			NAFL/D		
		Q1-Q4 N=514	Q5 N=117	*p*	Q1-Q4 N=297	Q5 N=75	*p*	Q1-Q4 N=244	Q5 N=62	*p*
Age		68.9 (4.1)	68.0 (4.4)	***0.045***	68.4 (3.9)	67.5 (4.3)	*0.100*	68.4 (4.0)	67.8 (4.2)	*0.185*
Sex, % male		54.9 (282)	55.6 (65)	*0.918*	53.2 (152)	48.0 (36)	*0.440*	50.8 (124)	40.3 (25)	*0.156*
Duration of diabetes, % ≤5years		40.5 (206)	31.9 (37)	*0.092*	42.0 (123)	27.0 (20)	***0.023***	42.0 (102)	27.9 (17)	*0.056*
	Diet	21.6 (111)	20.5 (24)	*0.901*	19.2 (57)	16.0 (12)	*0.619*	18.4 (45)	17.7 (11)	*1.000*
	Metformin	63.6 (327)	62.4 (73)	*0.833*	70.7 (210)	68.0 (51)	*0.673*	72.1 (176)	66.1 (41)	*0.352*
Diabetes treatment:	**Sulph.**	30.4 (156)	27.4 (32)	*0.576*	31.3 (93)	24.0 (18)	*0.259*	29.5 (72)	22.6 (14)	*0.343*
	TZD	16.1 (83)	23.9 (28)	*0.059*	17.8 (53)	20.0 (15)	*0.738*	19.7 (48)	22.6 (14)	*0.599*
	Insulin	13.6 (70)	13.7 (16)	*1.000*	12.8 (38)	16.0 (12)	*0.289*	12.7 (31)	17.7 (11)	*0.305*
Fasting glucose, mmol/L		6.88 (2.1)	7.10 (2.6)	*0.386*	7.18 (2.2)	7.46 (2.8)	*0.360*	7.11 (2.2)	7.40 (2.9)	*0.398*
HbA1c, %		7.15 (1.0)	7.37 (1.3)	*0.077*	7.29 (1.0)	7.56 (1.2)	*0.075*	7.28 (1.0)	7.56 (1.3)	*0.128*
Body mass index, kg/m^2		30.1 (4.6)	33.1 (6.0)	***<0.001***	31.0 (4.4)	33.8 (6.5)	***0.001***	31.0 (4.4)	34.2 (6.8)	***0.001***
Waist circumference, cm		104.0 (10.8)	110.6 (12.1)	***<0.001***	106.0 (10.3)	111.2 (12.6)	***0.001***	105.7 (10.4)	110.7 (13.3)	***0.007***
Total cholesterol, mmol/L		4.18 (0.8)	3.97 (0.6)	***0.032***	4.28 (0.8)	4.00 (0.6)	***0.005***	4.28 (0.9)	3.97 (0.6)	***0.007***
Triglycerides[a], mmol/L		0.16 (0.2)	0.19 (0.2)	*0.361*	0.23 (0.2)	0.25 (0.2)	*0.535*	0.24 (0.2)	0.25 (0.2)	*0.855*
Steatosis, % 'fatty'		44.6 (229)	26.5 (31)	*<0.001*	-	-	-	-	-	-
Excess alcohol intake[b]		11.1 (57)	20.5 (24)	***0.009***	11.4 (34)	22.7 (17)	***0.023***	-	-	-
Established hepatotoxic cause[c]		20.8 (107)	27.4 (32)	*0.138*	15.8 (47)	25.3 (19)	*0.063*	-	-	-

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic

HbA1c glycosylated haemoglobin; **LSM** liver stiffness measurement; **Sulph.** sulphonylurea; **TZD** thiozolidinedione

5.5.3 Surrogate markers of portal hypertension

In order to provide some validation of the highest quintiles of steatohepatitis and liver fibrosis markers containing the participants with the most advanced CLD associations with surrogate markers of portal hypertension were examined.

The top quintiles of each of the markers generally had significantly higher surrogate measures of portal hypertension than the lower four quintiles (Table 5-23).

Table 5-23 Surrogate markers of portal hypertension for quintiles. Values are mean (sd).

	Spleen size, cm			Platelet count, x10^9/L		
	Q1-4	**Q5**	***P***	**Q1-4**	**Q5**	***p***
CK18	10.1 (1.5)	10.6 (1.8)	***<0.001***	260 (69)	259 (64)	*0.887*
APRI	10.0 (1.4)	10.8 (1.8)	***<0.001***	275 (64)	200 (45)	***<0.001***
AST:ALT ratio	10.3 (1.4)	9.9 (1.8)	***0.019***	259 (67)	261 (71)	*0.808*
ELF	10.1 (1.5)	10.5 (2.0)	***0.037***	267 (70)	250 (60)	***0.022***
FIB4	10.1 (1.4)	10.6 (1.7)	***<0.001***	277 (62)	190 (42)	***<0.001***
HA	10.2 (1.5)	10.2 (1.9)	*0.867*	266 (69)	251 (69)	***0.031***
NFS	10.0 (1.4)	10.9 (1.6)	***<0.001***	275 (63)	197 (45)	***<0.001***
LSM	10.1 (1.5)	10.7 (1.6)	***<0.001***	262 (67)	252 (61)	*0.149*

5.5.4 Influence of chronic kidney disease and arthritis on liver markers

Liver markers were not significantly associated with other conditions known to affect circulating levels. With the exception of a significantly higher proportion of patients with chronic kidney disease in the highest NFS quintile (Table 5-24).

Table 5-24 Influence of chronic kidney disease and arthritis on markers. Values are % (n).

	Chronic kidney disease, %			Arthritis, %		
	Q1-4	**Q5**	***P***	**Q1-4**	**Q5**	***p***
CK18	18.1 (118)	19.8 (32)	*0.651*	35.0 (228)	41.4 (67)	*0.144*
APRI	20.1 (139)	17.3 (30)	*0.454*	37.8 (262)	32.9 (57)	*0.253*
AST:ALT ratio	18.4 (130)	23.2 (41)	*0.167*	37.4 (264)	35.6 (63)	*0.728*
ELF	15.8 (71)	24.1 (27)	*0.051*	37.3 (167)	45.5 (51)	*0.129*
FIB4	19.6 (136)	19.1 (33)	*0.915*	38.2 (265)	31.2 (54)	*0.094*
HA	17.4 (94)	19.4 (26)	*0.614*	36.7 (198)	37.3 (50)	*0.920*
NFS	16.8 (116)	29.1 (50)	***<0.001***	35.9 (248)	40.7 (70)	*0.252*
LSM	18.1 (93)	14.5 (17)	*0.419*	34.2 (176)	34.2 (40)	*1.000*

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; **HA** hyaluronic acid; **NAFLD** non-alcoholic fatty liver disease; **NFS** NAFLD Fibrosis Score; **LSM** liver stiffness measure.

5.6 Multivariable analysis

In order to investigate the association between steatohepatitis and liver fibrosis marker with potential risk factors after adjusting for confounders, I undertook multivariable analysis of each of the markers by each of the potential diabetes history and metabolic risk factors, adjusted for age, sex and established causes of liver disease.

The results of these analyses are shown in Table 5-25 to Table 5-32, both unadjusted (model 1) and adjusted for age, sex and established hepatotoxic causes (model 2). Each table is ordered by the risk factors explaining the greatest degree of variation after adjustment.

Risk factors maintaining statistical significance after adjustment varied for each marker. Most commonly, variables related to body fat (BMI and waist circumference) maintained statistical significance, for example with CK18, ELF, HA NFS and LSM.

Diabetes history related risk factors were not consistently related. In terms of diabetes treatment, higher CK18 and ELF levels were statistically significantly

associated with not being diet controlled (ie on a more intensive therapy), whereas higher APRI and FIB4 scores were significantly associated with having diet controlled diabetes. CK18 was the only marker significantly associated with OAHA use, with APRI, FIB4 and NFS being significantly associated with not using OAHA's. There were very few statistically significant associations after adjustment with fasting glucose, HbA1c and duration of diabetes, with the exception of the AST:ALT ratio which was statistically associated with better glucose control and a shorter duration of diabetes.

In general, the amount of variation in each of markers of steatohepatitis and liver fibrosis studied was poorly explained by any of the risk factors (R^2<15%). The only higher value was for the association between NFS and BMI (R^2 28%), however, it should be noted that BMI is part of the NFS formula.

Also of note, markers with similar compositions (APRI and FIB4) had similar statistically significant associations with risk factors.

Table 5-25 Multivariable association of risk factors with CK18. Values are standardised beta coefficients (95% CI)

	Model 1		*p*	R^2	Model 2		*p*	R^2
Hepatic steatosis	0.308	(0.241,0.377)	***<0.001***	0.095	0.299	(0.231,0.367)	***<0.001***	0.110
Triglycerides[a], mmol/L	0.149	(0.064,0.223)	***<0.002***	0.021	0.151	(0.066,0.226)	***<0.001***	0.054
Waist circumference, cm	0.135	(0.064,0.208)	***<0.001***	0.018	0.118	(0.047,0.193)	***0.001***	0.036
Body mass index, kg/m^2	0.095	(0.024,0.167)	***0.009***	0.009	0.090	(0.016,0.164)	***0.017***	0.030
Diet controlled	-0.082	(-0.150,-0.010)	***0.024***	0.007	-0.086	(-0.153,-0.014)	***0.019***	0.030
Any OAHA use	0.079	(0.008,0.149)	***0.030***	0.006	0.085	(0.014,0.155)	***0.019***	0.030
Fasting glucose, mmol/L	0.091	(0.019,0.160)	***0.013***	0.008	0.083	(0.012,0.152)	***0.022***	0.030
HbA1c, % or mmol/mol	0.077	(0.006,0.146)	***0.034***	0.006	0.069	(-0.003,0.139)	*0.060*	0.028
Total cholesterol, mmol/L	-0.038	(-0.121,0.044)	*0.363*	0.001	-0.043	(-0.127,0.042)	*0.324*	0.031
Insulin therapy	0.026	(-0.046,0.099)	*0.476*	0.001	0.022	(-0.050,0.095)	*0.546*	0.023
Duration of diabetes[a], years	-0.001	(-0.073,0.070)	*0.935*	-	0.005	(-0.067,0.076)	*0.897*	0.023

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

CK18 cytokeratin-18; **HbA1c** glycosylated haemoglobin; **OAHA** oral anti-hyperglycaemic agent.

Table 5-26 Multivariable association of risk factors with APRI. Values are standardised beta coefficients (95% CI)

	Model 1		*p*	R^2	Model 2		*p*	R^2
OAHA	-0.183	(-0.251,-0.115)	***<0.001***	0.034	-0.173	(-0.240,-0.107)	***<0.001***	0.080
Diet controlled	0.154	(0.086,0.221)	***<0.001***	0.024	0.141	(0.073,0.206)	***<0.001***	0.069
Duration of diabetes	-0.064	(-0.135,0.005)	*0.069*	0.004	-0.065	(-0.134,0.003)	*0.061*	0.055
Steatosis	0.053	(-0.016,0.124)	*0.130*	0.003	0.053	(-0.015,0.123)	*0.122*	0.053
HbA1c	-0.065	(-0.137,2.703)	*0.156*	0.004	-0.049	(-0.119,0.019)	*0.156*	0.055
Trig	0.026	(-0.053,0.106)	*0.513*	0.001	0.045	(-0.033,0.124)	*0.257*	0.054
Glucose	0.053	(-0.017,0.126)	*0.134*	0.003	0.044	(-0.025,0.115)	*0.209*	0.053
Cholesterol	-0.064	(-0.142,0.015)	*0.115*	0.004	-0.035	(-0.114,0.044)	*0.388*	0.053
Waist	0.003	(-0.067,0.073)	*0.936*	-	-0.013	(-0.083,0.057)	*0.718*	0.053
BMI	-0.034	(-0.105,0.036)	*0.338*	0.001	0.009	(-0.062,0.081)	*0.794*	0.052
Insulin	-0.009	(-0.080,0.061)	*0.797*	-	0.003	(-0.066,0.072)	*0.935*	0.050

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

APRI aspartate to platelet ratio index;; **HbA1c** glycosylated haemoglobin; **OAHA** oral anti-hyperglycaemic agent.

Table 5-27 Multivariable association of risk factors with AST:ALT ratio. Values are standardised beta coefficients (95% CI)

	Model 1		*p*	R^2	Model 2		*p*	R^2
Steatosis	-0.171	(-0.242,-0.105)	***<0.001***	0.029	-0.157	(-0.225,-0.092)	**<0.001**	0.104
HbA1c	-0.157	(-0.221,-0.088)	***<0.001***	0.025	-0.128	(-0.191,-0.061)	**<0.001**	0.096
Glucose	-0.140	(-0.203,-0.070)	***<0.001***	0.020	-0.124	(-0.185,-0.056)	**<0.001**	0.095
Duration of diabetes	0.085	(0.016,0.154)	***0.015***	0.007	0.088	(0.022,0.155)	**0.009**	0.090
Cholesterol	0.108	(0.029,0.180)	***0.007***	0.012	0.083	(0.005,0.155)	**0.037**	0.076
Waist	-0.122	(-0.192,-0.054)	***<0.001***	0.015	-0.074	(-0.143,-0.007)	**0.032**	0.084
BMI	-0.069	(-0.140,-0.001)	***0.046***	0.005	-0.056	(-0.126,0.012)	0.108	0.083
Triglycerides	-0.073	(-0.148,0.006)	***0.071***	0.005	-0.052	(-0.126,0.024)	0.185	0.072
OAHA	-0.043	(-0.111,0.025)	*0.218*	0.002	-0.030	(-0.096,0.036)	0.370	0.080
Insulin	0.014	(-0.056,0.084)	*0.691*	-	0.026	(-0.041,0.094)	0.442	0.080
Diet	0.016	(-0.052,0.084)	*0.646*	-	0.000	(-0.066,0.065)	0.989	0.079

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

ALT alanine aminotransferase; **AST** aspartate aminotransferase; HbA1c glycosylated haemoglobin; **OAHA** oral anti-hyperglycaemic agent.

Table 5-28 Multivariable association of risk factors with ELF. Values are standardised beta coefficients (95% CI)

	Model 1		*p*	R^2	Model 2		*p*	R^2
Body mass index, kg/m^2	0.135	(0.048,0.214)	***0.002***	0.018	0.171	(0.084,0.247)	***<0.001***	0.132
Insulin therapy	0.147	(0.060,0.228)	***0.001***	0.022	0.158	(0.075,0.234)	***<0.001***	0.130
Waist circumference, cm	0.085	(-0.002,0.169)	*0.055*	0.007	0.142	(0.058,0.222)	***0.001***	0.124
Duration of diabetes[a], years	0.149	(0.059,0.222)	***0.001***	0.022	0.124	(0.040,0.197)	***0.003***	0.120
Diet controlled	-0.109	(-0.186,-0.021)	***0.014***	0.012	-0.114	(-0.187,-0.030)	***0.007***	0.118
HbA1c, % or mmol/mol	0.047	(-0.037,0.128)	*0.284*	0.002	0.077	(-0.006,0.152)	*0.070*	0.112
Any OAHA use	0.065	(-0.021,0.148)	*0.141*	0.004	0.067	(-0.015,0.147)	*0.110*	0.109
Fasting glucose, mmol/L	0.000	(-0.083,0.82)	*0.992*	-	0.016	(-0.064,0.094)	*0.711*	0.106
Total cholesterol, mmol/L	0.019	(-0.081,0.117)	*0.719*	-	-0.012	(-0.111,0.088)	*0.823*	0.092
Hepatic steatosis	-0.030	(-0.113,0.054)	*0.491*	0.001	0.011	(-0.070,0.091)	*0.802*	0.105
Triglycerides[a], mmol/L	-0.026	(-0.120,0.072)	*0.622*	0.001	0.003	(-0.091,0.097)	*0.947*	0.090

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

ELF European Liver Fibrosis panel; **HbA1c** glycosylated haemoglobin; **OAHA** oral anti-hyperglycaemic agent.

Table 5-29 Multivariable association of risk factors with FIB4. Values are standardised beta coefficients (95% CI)

	Model 1		*p*	R^2	Model 2		*p*	R^2
Any OAHA use	-0.170	(-0.240,-0.103)	***<0.001***	0.029	-0.154	(-0.221,-0.090)	***<0.001***	0.124
Diet controlled	0.141	(0.073,0.209)	***<0.001***	0.020	0.119	(0.053,0.184)	***<0.001***	0.114
HbA1c, % or mmol/mol	-0.111	(-0.183,-0.043)	***0.002***	0.012	-0.067	(-0.135,0.000)	*0.050*	0.107
Hepatic steatosis	-0.061	(-0.132,0.009)	*0.085*	0.004	-0.040	(-0.108,0.027)	*0.236*	0.102
Fasting glucose, mmol/L	0.024	(-0.047,0.096)	*0.500*	0.001	0.029	(-0.039,0.098)	*0.393*	0.100
Total cholesterol, mmol/L	-0.048	(-0.126,0.031)	*0.232*	0.002	-0.028	(-0.105,0.049)	*0.477*	0.093
Waist circumference, cm	-0.041	(-0.113,0.028)	*0.240*	0.002	-0.015	(-0.084,0.054)	*0.666*	0.101
Triglycerides[a], mmol/L	-0.029	(-0.109,0.050)	*0.467*	0.001	0.011	(-0.066,0.088)	*0.786*	0.092
Duration of diabetes[a], years	-0.001	(-0.071,0.070)	*0.988*	-	-0.006	(-0.074,0.061)	*0.850*	0.104
Insulin therapy	-0.020	(-0.092,0.050)	*0.564*	-	0.002	(-0.066,0.070)	*0.960*	0.100
Body mass index, kg/m^2	-0.074	(-0.148,-0.005)	***0.035***	0.005	0.000	(-0.070,0.071)	*0.991*	0.102

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

FIB4 Fibrosis-4 Score; **HbA1c** glycosylated haemoglobin; **OAHA** oral anti-hyperglycaemic agent.

Table 5-30 Multivariable association of risk factors with HA. Values are standardised beta coefficients (95% CI)

	Model 1		*p*	R^2	Model 2		*p*	R^2
Body mass index, kg/m^2	0.057	(-0.022,0.137)	*0.156*	0.003	0.115	(0.039,0.195)	***0.003***	0.118
Waist circumference, cm	0.029	(-0.050,0.110)	*0.462*	0.001	0.098	(0.022,0.178)	***0.012***	0.115
Duration of diabetes[a], years	0.091	(0.013,0.170)	***0.023***	0.008	0.071	(-0.004,0.146)	*0.065*	0.112
Insulin therapy	0.060	(-0.019,0.144)	*0.132*	0.004	0.069	(-0.006,0.150)	*0.070*	0.110
Diet controlled	-0.054	(-0.131,0.024)	*0.176*	0.003	-0.068	(-0.141,0.007)	*0.076*	0.109
Any OAHA use	0.045	(-0.034,0.125)	*0.257*	0.002	0.055	(-0.019,0.132)	*0.145*	0.099
HbA1c, % or mmol/mol	0.019	(-0.059,0.097)	*0.631*	-	0.052	(-0.023,0.127)	*0.173*	0.109
Total cholesterol, mmol/L	-0.026	(-0.119,0.066)	*0.576*	0.001	-0.048	(-0.138,0.042)	*0.296*	0.115
Triglycerides[a], mmol/L	-0.079	(-0.163,0.013)	*0.096*	0.006	-0.046	(-0.129,0.041)	*0.315*	0.114
Fasting glucose, mmol/L	-0.049	(-0.127,0.029)	*0.221*	0.002	-0.028	(-0.103,0.047)	*0.461*	0.107
Hepatic steatosis	-0.036	(-0.115,0.043)	*0.370*	0.001	-0.003	(-0.079,0.073)	*0.938*	0.105

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

HA hyaluronic acid; **HbA1c** glycosylated haemoglobin; **OAHA** oral anti-hyperglycaemic agent.

Table 5-31 Multivariable association of risk factors with NFS. Values are standardised beta coefficients (95% CI)

	Model 1		*p*	R^2	Model 2		*p*	R^2
Body mass index, kg/m^2	0.422	(0.366,0.494)	***<0.001***	0.178	0.504	(0.453,0.577)	***<0.001***	0.284
Waist circumference, cm	0.394	(0.332,0.461)	***<0.001***	0.155	0.433	(0.373,0.500)	***<0.001***	0.225
Total cholesterol, mmol/L	-0.148	(-0.226,-0.069)	***<0.001***	0.022	-0.142	(-0.221,-0.062)	***<0.001***	0.063
Any OAHA use	-0.088	(-0.157,-0.019)	***0.013***	0.008	-0.078	(-0.146,-0.010)	***0.025***	0.052
Diet controlled	0.060	(-0.009,0.128)	*0.089*	0.004	0.045	(-0.023,0.113)	*0.196*	0.048
Triglycerides[a], mmol/L	0.018	(-0.062,0.099)	*0.651*	-	0.044	(-0.035,0.125)	*0.271*	0.046
Fasting glucose, mmol/L	0.031	(-0.040,0.103)	*0.384*	0.001	0.034	(-0.036,0.105)	*0.334*	0.047
HbA1c, % or mmol/mol	-0.059	(-0.130,0.010)	*0.092*	0.004	-0.028	(-0.097,0.041)	*0.431*	0.040
Duration of diabetes[a], years	0.020	(-0.050,0.090)	*0.577*	-	0.015	(-0.053,0.085)	*0.656*	0.047
Hepatic steatosis	-0.015	(-0.085,0.055)	*0.669*	-	-0.001	(-0.070,0.068)	*0.979*	0.046
Insulin therapy	-0.016	(-0.088,0.055)	*0.651*	-	-0.001	(-0.071,0.069)	*0.983*	0.046

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

HbA1c glycosylated haemoglobin; **NFS** NAFLD Fibrosis Score; **OAHA** oral anti-hyperglycaemic agent.

Table 5-32 Multivariable association of risk factors with LSM. Values are standardised beta coefficients (95% CI)

	Model 1		*p*	R^2	Model 2		*p*	R^2
Body mass index, kg/m^2	0.221	(0.164,0.345)	***<0.001***	0.049	0.235	(0.177,0.364)	***<0.001***	0.066
Waist circumference, cm	0.219	(0.162,0.344)	***<0.001***	0.048	0.220	(0.162,0.344)	***<0.001***	0.061
Hepatic steatosis	0.196	(0.122,0.288)	***<0.001***	0.038	0.195	(0.121,0.288)	***<0.001***	0.052
HbA1c, % or mmol/mol	0.127	(0.047,0.211)	***0.002***	0.016	0.132	(0.051,0.217)	***0.002***	0.032
Triglycerides[a], mmol/L	0.099	(0.004,0.157)	***0.038***	0.010	0.105	(0.009,0.162)	***0.029***	0.040
Total cholesterol, mmol/L	-0.077	(-0.144,0.014)	*0.109*	0.006	-0.084	(-0.152,0.010)	*0.085*	0.034
Fasting glucose, mmol/L	0.083	(0.001,0.170)	***0.046***	0.007	0.076	(-0.006,0.163)	*0.069*	0.021
Duration of diabetes[a], years	0.033	(-0.050,0.118)	*0.426*	0.001	0.039	(-0.044,0.125)	*0.348*	0.017
Diet controlled	-0.025	(-0.104,0.055)	*0.545*	0.001	-0.034	(-0.114,0.046)	*0.408*	0.016
Any OAHA use	0.022	(-0.059,0.104)	*0.590*	-	0.029	(-0.052,0.111)	*0.480*	0.015
Insulin therapy	0.016	(-0.070,0.105)	*0.696*	-	0.017	(-0.069,0.106)	*0.676*	0.015

[a] Analysed on the Log10 scale

Model 1 - Unadjusted model, individual variables with no adjustment

Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies

HbA1c glycosylated haemoglobin; **LSM** liver stiffness measure; **OAHA** oral anti-hyperglycaemic agent.

5.7 Sensitivity analysis using imputed data

Due to the relatively large number of subjects in whom ELF (and to a lesser extent, CK18) data were unavailable (i.e. 'missing' at random) (see section 5.1.2), a sensitivity analysis was undertaken using multiple imputation (imputation dataset included all 888 subjects eligible for inclusion in the analyses, Figure 5-3).

Standard procedures were followed with multiple imputation using chained equations performed on the liver sub-study data set using variables associated with missingness (CK18 and sex) and variables highly correlated with the missing variables. Options available included using all anticipated analysis and outcome variables in a single imputation model or developing individual imputed datasets specific to the analysis of interest. It was decided to run an exploratory imputation model to allow sensitivity analyses of the descriptive analyses of liver injury markers (section 5.8) compared with the original dataset and then to proceed with individualised imputed datasets as needed for analysis.

For the initial imputation model the following variables measured at the liver sub-study were included: alcohol excess, ALP, ALT, AST, bilirubin, BMI (at baseline), CK18, diabetes treatment type, diastolic and systolic blood pressures, duration of diabetes, ELF, fasting glucose, ferritin, GGT, HA (at BL), HbA1c, HDL-cholesterol, hepatic steatosis, hepatotoxic medication use, LDL-cholesterol, platelets (at baseline), prevalent liver disease, sex, smoking status, strongly positive autoantibodies, total cholesterol, triglycerides, and waist circumference. Ten iterations and 100 datasets were permitted. Conditional limits were applied to the imputed values: CK18 – minimum 0 and maximum 1000 (assay upper acceptable value), ELF minimum 0 and maximum unrestricted.

Prior to imputation the median CK18 score (n=860) was 102.8 (IQR 76.9-138.3) and after imputation the pooled mean of median values (n-939) was 107.9 (sd 0.9). For ELF, the prior (n=683) mean was 8.90 (sd 0.8), and after imputation the pooled mean

(n=939) was 8.96. Given that following imputation the summary values were preserved it was felt the imputed dataset was suitable for further use in the thesis.

The results confirmed those in the original dataset with only minimal differences found in effect sizes and significance levels (Table 5-33 and Table 5-34).

Table 5-33 Imputed data CK18 (U/L) associations. Values are correlation coefficients or mean (SEM).

		All	*p*	Steatosis	*p*	NAFL/D	*p*
Age		0.175	***<0.001***	-0.057	*0.228*	-0.073	*0.159*
Sex	**Male**	109.6 (1.1)	-	131.8 (2.6)	*0.113*	128.8 (2.6)	-
	Female	107.2 (1.1)	*0.484*	120.2 (2.4)		117.5 (2.3)	*0.160*
Duration of diabetes		0.003	*0.939*	0.026	*0.591*	0.023	*0.675*
Fasting glucose		0.102	***0.005***	0.125	***0.010***	0.084	*0.118*
HbA1c		0.075	*0.045*	0.080	*0.098*	0.068	*0.207*
Treatment:	**Diet**	100.0 (2.0)	-	112.2 (3.4)	-	112.2 (3.4)	-
	OAHA	109.6 (1.1)	*0.102*	125.9 (2.5)	*0.087*	123.0 (2.5)	*0.233*
	Insulin	112.2 (3.4)	*0.115*	13.4.9 (5.4)	*0.073*	125.9 (5.0)	*0.269*
Body mass index		0.118	***0.001***	0.035	*0.462*	0.078	*0.142*
Waist circumference		0.145	***<0.001***	0.084	*0.082*	0.128	***0.016***
Total cholesterol		-0.064	*0.154*	-0.050	*0.375*	-0.077	*0.222*
Triglycerides[a]		0.131	***0.002***	0.061	*0.272*	0.097	*0.108*
Alcohol excess[b]**:**	**No**	107.2 (1.1)	-	123.0 (1.2)	-	NA	-
	Yes	125.9 (3.8)	***0.006***	147.9 (1.5)	***0.015***		
Established hepatotoxic cause[c]**:**	**No**	122.7 (0.05)	-	142.6 (0.06)	-	NA	-
	Yes	106.7 (0.02)	***0.005***	121.5 (0.03)	***0.013***		

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic
CK18 cytokeratin-18; **NAFL/D** non-alcoholic fatty liver /disease

Table 5-34 Imputed data ELF associations. Values are correlation coefficients or mean (SEM)

		All	*p*	Hepatic steatosis	*p*	NAFL/D	*p*
Age		0.267	***<0.001***	0.235	***<0.001***	0.211	***0.001***
Sex:	**Male**	9.01 (0.04)	***0.015***	8.86 (0.06)	*0.119*	8.90 (0.06)	*0.174*
	Female	8.87 (0.04)		8.98 (0.05)		9.01 (0.06)	
Duration of diabetes		0.137	***<0.001***	0.152	***0.002***	0.157	***0.005***
Fasting glucose		0.015	*0.691*	-0.009	*0.861*	-0.029	*0.612*
HbA1c		0.052	*0.175*	0.017	*0.725*	0.020	*0.722*
Diabetes treatment:	**Diet**	8.75 (0.06)	-	8.79 (0.09)	-	8.81 (0.10)	-
	OAHA	8.94 (0.04)	***0.013***	8.89 (0.05)	*0.321*	8.92 (0.05)	*0.306*
	Insulin	9.17 (0.09)	***<0.001***	9.25 (0.10)	***0.001***	9.26 (0.11)	***0.002***
BMI		0.136	***<0.001***	0.183	***<0.001***	0.204	***<0.001***
Waist circumference		0.094	***0.013***	0.133	***0.007***	0.159	***0.003***
Total cholesterol		-0.028	*0.551*	-0.058	*0.331*	-0.057	*0.388*
Triglycerides[a]		-0.006	*0.897*	-0.071	*0.255*	-0.072	*0.298*
Alcohol excess[b]**:**	**No**	8.97 (0.03)	-	8.95 (0.04)	-	NA	-
	Yes	8.69 (0.09)	***0.003***	8.75 (0.11)	*0.068*		
Established hepatotoxic cause[c]**:**	**No**	8.90 (0.03)	-	8.89 (0.04)	-	NA	-
	Yes	8.71 (0.08)	***0.025***	8.69 (0.09)	***0.046***		

[a] Analysed on the Log10 scale; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as alcohol excess or ASmA titer >1:160 or AMA titer >1:40; [d] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic
ELF Enhanced Liver Fibrosis pamel; **NAFL/D** non-alcoholic fatty liver /disease

5.8 Liver fibrosis marker agreement

On the assumption that all liver fibrosis markers are trying to diagnose the same underlying pathology and in order to try and identify a group of participants with probable liver fibrosis I analysed the agreement between five markers of liver fibrosis (APRI, AST:ALT ratio, ELF, FIB4 and LSM). These markers were chosen because they were developed based on a range differing biological plausibilities and accessibility in clinical practice: AST:ALT ratio – non-specific liver injury; APRI and FIB4 – combining non-specific liver injury and portal hypertension; ELF - extra-cellular matrix based markers; and LSM - imaging.

For this analysis, I used the follow up ET2DS study population, since one of the key markers (LSM) was not available from earlier phases of the study. The 831 subjects attending the 4 year follow up clinic therefore formed the study population for the analysis presented in this section. As noted previously, differences between attenders at baseline and at the follow-up clinic were minimal. In addition, eleven patients were excluded from the analysis due to pre-diagnosed liver disease. 767 subjects had a complete dataset for APRI, AST:ALT ratio, ELF and FIB4 (proportion missing data, APRI 2.9%, AST/ALT ratio 1.8%, ELF 3.1% and FIB4 4.2%, all missing at random), and of these LSM was reported for 648 subjects.

A total of 282 subjects (248 for LSM) fulfilled the criteria for NAFL/D and had a complete fibrosis marker dataset.

LSM was missing in 119 subjects (15.3%) due to a) inability to obtain readings as described in Section 5.1.2. In brief, participants with missing LSM were significantly more obese, with poorer glucose control and had higher measres of all non-specific liver injury markers (ALT, AST and GGT).

5.8.1 Correlations

Correlations between the markers are shown in Table 5-35 (associated scatter plots in Appendix L). After adjustment for age and sex, correlation was strong between APRI and FIB4 ($r=0.92$) but all others were ≤ 0.5. The inter-item correlation for all five markers was just below acceptable ($\alpha=0.67$) with minimal improvement with the removal of any marker. Correlations were similar in the NAFL/D cohort (Table 5-35B).

Table 5-35 Correlation between markers. Values are correlation coefficients, adjusted for age and sex

A. Full cohort

APRI	r=0.30			
ELF	r=0.24	r=0.31		
FIB4	r=0.53	r=0.92	r=0.30	
LSM	r=0.15	r=0.20	r=0.25	r=0.16
	AST/ALT	APRI	ELF	FIB4

B. NAFL/D cohort

APRI	r=0.32			
ELF	r=0.18	r=0.32		
FIB4	r=0.53	r=0.93	r=0.29	
LSM	r=0.15	r=0.29	r=0.26	r=0.29
	AST/ALT	APRI	ELF	FIB4

APRI aspartate aminotransferase-platelet ratio index; **AST/ALT** aspartate aminotransferase-alanine aminotransferase ratio; **ELF** European Liver Fibrosis panel; **FIB4** Fibrosis-4 Score; **LSM** liver stiffness measure.
All p<0.001

5.8.2 'Validated' cut-offs

Literature based cut-offs yielded marked differences in the proportions of the cohort with probable liver fibrosis in the full cohort: APRI 0.8%, AST/ALT ratio 22.4%, ELF 7.0%, FIB4 68.3%, and LSM 4.5%. And in the NAFL/D subgroup: APRI 0.4%, AST/ALT ratio 16.7%, ELF 4.3%, FIB4 63.8%, and LSM 4.8%.

5.8.3 Highest/lowest percentile agreement

Agreement between the top 5% of the distribution for each marker pair was poor (Table 5-36). APRI and FIB4 had the best positive agreement at 76.4%, but agreement for all of the other marker pairs was between 9% and 32%. When the comparison groups were altered to reflect the top 3% and 7% of the marker distributions, then with the exception of APRI and FIB4, the agreement did not alter greatly (agreement 9-26% and 11-38% respectively). When analyses were restricted to the study population with NAFL/D, agreement between the top 10% of the distribution for each marker pair remained generally poor (agreement for APRI and FIB4 was 68%, all other pairs between 14% and 36%), as did agreement for the top 5% and 15% of the distributions (agreements from 0% to 36% and from 21% to 36% respectively, with exception of APRI/FIB4).

Table 5-36 Agreement between marker pairs for the top 5% or 10% of values, in the full cohort and non-alcoholic fatty liver /disease subgroup.

A. Positive agreement (presence of fibrosis) in the top 5% of the full cohort.

APRI	18.4%			
ELF	18.4%	31.6%		
FIB4	34.2%	76.3%	34.2%	
LSM	9.5%	12.7%	15.9%	12.7%
	AST:ALT ratio	APRI	ELF	FIB4

B. Negative agreement (absence of fibrosis) in the bottom 95% of the full cohort.

APRI	95.7%			
ELF	95.7%	96.4%		
FIB4	96.6%	98.8%	91.8%	
LSM	95.4%	95.5%	95.7%	95.5%
	AST:ALT ratio	APRI	ELF	FIB4

C. Positive agreement (presence of fibrosis) in the top 10% of the non-alcoholic fatty liver /disease subgroup.

APRI	28.6%			
ELF	14.3%	28.6%		
FIB4	35.7%	67.9%	32.1%	
LSM	29.2%	20.8%	25.0%	25.0%
	AST:ALT ratio	APRI	ELF	FIB4

D. Negative agreement (absence of fibrosis) in the bottom 90% of the non-alcoholic fatty liver /disease subgroup.

APRI	92.1%			
ELF	90.6%	92.1%		
FIB4	92.9%	96.5%	92.5%	
LSM	92.4%	91.5%	9.9%	91.9%
	AST:ALT ratio	APRI	ELF	FIB4

APRI aspartate aminotransferase-platelet ratio index; **AST:ALT ratio** aspartate aminotransferase-alanine aminotransferase ratio; **ELF** European Liver Fibrosis panel; **FIB4** Fibrosis-4 Score; **LSM** liver stiffness measure.

The top viginile (decile in NAFL/D) was suggestive of patients with advanced liver fibrosis with clinical data implicating advanced fibrosis/cirrhosis with platelet counts being significantly lower and spleen size being significantly larger in the majority of cases (Appendix L).

Negative agreement (agreement on the absence of fibrosis) was more consistent for both the full cohort analysis and the NAFL/D subgroup (90-99%) with minimal change with the alternative cut-offs described. 2x2 tables are available in the supplementary material (Appendix L).

5.9 Summary

In this chapter, I found that potential markers of steatohepatitis and liver fibrosis had varied relationships with diabetes history. AST:ALT ratio, ELF, FIB4, and LSM were statistically associated with hyperglycaemia, and ELF and HA with increasing duration of diabetes. Elevated ELF was associated with OAHA and insulin use and APRI, FIB4 and NFS with diet control. Most commonly, elevated markers of steatohepatitis and liver fibrosis were associated with older age and higher body fat measures (BMI and waist circumference). However, most of these relationships between liver markers and body fat measures lost statistical significance when limiting the population to only those with hepatic steatosis and/or NAFL/D. The presumption that poorer diabetes control (indicated by higher HbA1c) would be related to elevated steatohepatitis and fibrosis markers was not proven.

CHAPTER 6 Results II: clinically significant chronic liver disease in older people with type 2 diabetes – prevalence, incidence and associated risk factors

Several studies have found type 2 diabetes to be an independent risk factor for the progression of NAFL/D[109,345]. However, data on the progression to cirrhosis and HCC in community-based patients with diabetes is limited. The ability to identify patients with and at risk of developing such clinically significant chronic liver disease would promote early intervention strategies and guide clinical follow-up, ensuring timely detection of cirrhosis where assimilation into surveillance programmes for HCC and varices as well as screening for cardiovascular disease has been shown to improve patient outcomes[113,171-173]. Given the high prevalence of abnormal liver enzyme tests and of hepatic steatosis within the type 2 diabetic population[165,324] there is a pressing clinical need to identify those who are most likely to progress, as well as those who are not, in order to reduce the costs and anxiety resulting from unnecessary investigations or monitoring.

Within the diabetic population annual liver enzyme checks are commonly performed, but there are no clear and consistent national guidelines on their application and interpretation, particularly in the community setting.

Given that I was unable to identify an accurate and reliable non-invasive measure of sub-clinical CLD in the pre-ceding chapter, the current chapter focuses on CLD identified clinically through standard clinical investigations as the outcome of interest. Moreover, I aimed to investigate whether any of the non-invasive markers of sub-clinical CLD presented in the preceding chapter were able to discriminate between subjects who did, and did not, subsequently develop clinically significant CLD.

In this chapter I have:

(i) described the outcome of extensive liver related investigations amongst a population at high risk of CLD (older people with type 2 diabetes)

(ii) described the prevalence (known and unknown) and incidence of clinically significant CLD amongst older people with type 2 diabetes.

(iii) determined the association of metabolic and CLD related risk factors with prevalent and incident clinically significant CLD in type 2 diabetes.

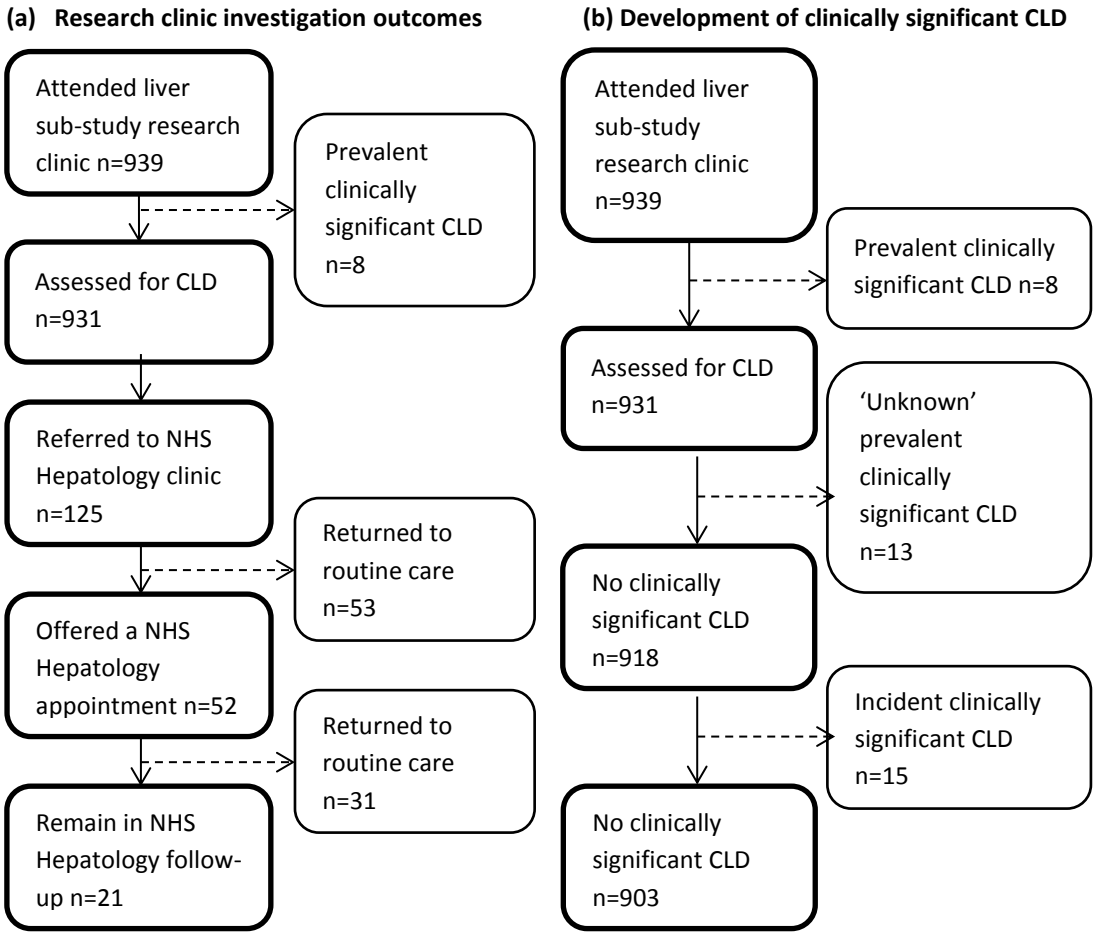
6.1 Follow-up data for clinical chronic liver disease

In order to investigate clinically significant CLD, clinical CLD follow-up data was available in some form for all 939 liver sub-study clinic attenders, including non-attenders at the follow-up clinic. All 831 attenders at follow-up completed a patient self-report questionnaire. Of the non-attenders, 147 were still alive, of which 43 had a GP questionnaire returned. All 939 participants had data linkage information on hospital discharges related to CLD available. Subject flow is shown in Figure 6-1A

6.2 Outcomes of liver related investigations

Prior to the initial liver sub-study research clinic eight patients had known prevalent clinically significant CLD (ALD n=5, NAFLD cirrhosis n=2, primary biliary cirrhosis n=1), and were therefore excluded from the investigation of unknown prevalent disease (Figure 6-1B).

Figure 6-1 Subject flow in the investigation and diagnosis of clinically significant CLD



CLD chronic liver disease; **NHS** National Health Service

Following the liver sub-study research clinic investigations 13.4% (125/931) subjects met the study protocol criteria as high-risk for the presence of liver disease (section 4.4.7) and were referred to see a consultant Hepatologist (referral reasons: positive autoantibodies n=84, abnormal liver enzymes n=46, elevated HA or AST:ALT n=44, enlarged spleen n=11, low platelets n=7, elevated ferritin, alpha-feto protein or positive viral serology n=6, cirrhosis on USS n=1). Subjects meeting the criteria for referral were significantly more likely to be female (59.2 vs 46.3 %, p=0.009), have poorer diabetes control (mean fasting glucose 7.60 mmol/L (sd 3.0) vs 6.76 mmol/L (2.2) p=0.003, mean HbA1c 7.37 % (sd 1.2) vs 7.17 % (1.1) p=0.047), higher total cholesterol (mean 4.3 mmol/L (sd 0.8) vs 4.1 mmol/L (0.8) p=0.024) and were more

likely to have a known risk factor for CLD (alcohol excess 20.8 % vs 11.6% p=0.006, positive autoantibodies 3.2 % vs 0.4% p=0.010) (Table 6-1).

Of those subjects who met the specialist Hepatology referral criteria, 52 (41.6%) were offered an appointment at an NHS Hepatology clinic and the remainder returned to standard care after triage by a consultant Hepatologist. The referrals were split between two consultant Hepatologists who reviewed the triggering result(s) and considered it/them within the clinical context. The majority of those not seen had low level titres of positive autoantibodies or isolated mildly elevated liver enzymes only. Similarly to those referred, those actually seen had significantly poorer diabetes control (mean fasting glucose 7.93 mmol/L (sd 3.3) vs 6.81 mmol/L (2.2) p=0.021, mean HbA1c 7.69 % (sd 1.3) vs 7.17 % (1.0) p=0.008) and were more likely to have a known risk factor for CLD (34.6% vs 20.4% p=0.022) (Table 6-1).

Of those referrals seen, 31 (59.6%) were immediately discharged following initial assessment on the basis of having either simple fatty liver with low risk for the presence of significant liver fibrosis and/or false positive indicators of clinically significant CLD. Twenty-one subjects (40.4%; NAFL/D n=16, mixed ALD/NAFL/D n=2, ALD n=1, primary biliary cirrhosis n=1, hepatitis B viruS n=1) remain under active Hepatology follow-up.

Table 6-1 Study population. Values are mean (sd), median (IQR) or % (n).

		No known prevalent clinically significant CLD n=923	Met Hepatology referral criteria n=125	*p*	Seen by Hepatology services n=52	*p*
Age, years		68.9 (4.2)	69.3 (4.3)	*0.224*	69.3 (4.6)	*0.423*
Sex, % male		52.3% (483)	40.8 (51)	***0.007***	40.4 (21)	*0.087*
SIMD quintile, %	**I**	11.4 (105)	9.6 (12)	-	9.6 (5)	-
	V	34.3 (317)	31.2 (39)	***0.041***	25.0 (13)	***0.031***
Random glucose, mmol/L		6.89 (2.3)	7.60 (3.0)	***0.003***	7.93 (3.3)	***0.021***
HbA1c, %		7.20 (1.1)	7.37 (1.2)	***0.046***	7.69 (1.3)	***0.008***
Duration of diabetes, % <5 years		25.7 (235)	20.0 (25)	*0.124*	17.3 (9)	*0.191*
Diabetes treatment:	**Diet, %**	19.5 (180)	19.2 (24)	*1.000*	19.2 (10)	*1.000*
	OAHA, %	64.8 (598)	63.2 (79)	*0.688*	59.6 (31)	*0.456*
	Insulin, %	15.7 (145)	17.6 (22)	*0.511*	21.2 (11)	*0.245*
BMI, kg/m^2		31.3 (5.7)	31.7 (6.1)	*0.411*	32.0 (6.6)	*0.414*
Total cholesterol, mmol/L		4.4 (0.8)	4.3 (0.8)	***0.021***	4.1 (0.8)	*0.690*
Triglycerides, mmol/L		1.66 (0.9)	1.74 (1.0)	*0.305*	1.50 (0.6)	*0.073*
sBP, mmHg		138.2 (18.5)	140.6 (18.6)	*0.115*	138.7 (17.1)	*0.845*
Established hepatotoxic cause[a], %		20.9 (193)	31.2 (39)	***0.004***	34.6 (18)	***0.021***
Alcohol excess[b], %		12.8 (118)	20.8 (26)	***0.006***	21.2 (11)	*0.084*
Hepatotoxic medication[c], %		9.2 (6)	10.4 (13)	*0.618*	11.5 (6)	*0.467*
Positive autoantibodies[d], %		0.7 (6)	3.2 (4)	***0.005***	3.8 (2)	***0.046***

BMI body mass index; **IQR** inter-quartile range; **OAHA** oral anti-hyperglycaemic agent; **sBP** systolic blood pressure; **sd** standard deviation; **SIMD** Scottish Index of Multiple Deprivation

[a] Defined as any of b-d below; [b] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem; [c] Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the liver assessment; [d] Defined as ASMA titre >1:160 or AMA titre >1:40

6.3 Association of potential risk factors with unknown prevalent clinically significant chronic liver disease

In addition to the known eight prevalent clinically significant CLD cases, as a consequence of the liver sub-study investigations, 13 (1.4%) patients were diagnosed with previously unknown prevalent clinically significant CLD (including one HCC, the remainder cirrhosis). The reasons for their initial referral were wide-ranging and are shown in Table 6-2. All patients with unknown prevalent clinically significant CLD had abnormal liver enzymes, and each had between 2 and 6 of the referral criteria.

Whilst a the presence of abnormal LFTs or an elevated HA had a high specificity for detecting unknown clinically significant CLD they were associated with a low (<10%) PPV, meaning large numbers of false positives were detected. The best combination of the most sensitive of the routinely measured markers only improved the PPV to 48%.

Risk factors with a statistically significant association with unknown prevalent cases were: elevated liver enzymes (but not ALT), inflammatory markers (CRP, IL6, TNFα), steatohepatitis markers (CK18) and liver fibrosis markers (APRI, AST:ALT ratio, ELF, FIB4, HA, NFS) (all $p < 0.01$, Table 6-3 and Table 6-4). Given these associations the 'validated' cut-offs for NAFLD, as described in previous chapters, were trialled as diagnostic tests (Table 6-5). As previously the PPV of the tests remain poor resulting in large numbers of false positive results.

Overall the prevalence of clinically significant CLD was 2.2% (known plus unknown).

Table 6-2 Diagnostic accuracy of referral criteria, and combinations, for unknown prevalent clinically significant chronic liver disease

	Number with referral reason	*Sens., %*	*Spec., %*	*PPV, %*	*NPV, %*
Any abnormal LFT[a]	13	**100**	84	8	100
Abnormal ALT	*3*	(72-100)	(80-85)	(5-14)	(99-1)
Abnormal AST	*9*				
Abnormal GGT	*9*				
Elevated HA[b]	8	89 (51-99)	51 (47-55)	2 (1-5)	99 (98-100)
AST:ALT >1	10	**77** (46-94)	64 (61-67)	3 (2-6)	99 (98-100)
Positive autoantibodies	1	8 (0-38)	99 (99-100)	17 (1-64)	99 (98-99)
Platelets <150x10^9/l	6	46 (20-74)	97 (96-98)	18 (7-35)	99 (98-100)
Cirrhosis on USS	1	8 (0-38)	100 (99-100)	100 (5-100)	99 (98-99)
Spleen >13cm	9	**69** (39-90)	96 (94-97)	20 (10-34)	99 (98-100)
Significant steatosis on USS	6	46 (20-74)	43 (40-46)	1 (0-3)	98 (97-100)
Any abnormal LFT[a] **+ 1 of AST:ALT>1 or platelets <150x10^9/l**	11	85 (54-97)	95 (93-96)	18 (10-31)	99 (99-100)
Any abnormal LFT[a] **+ 1 of platelets<150x10^9/l or spleen >13cm**	10	77 (46-94)	99 (98-99)	**48** (26-70)	99 (99-100)

[a] defined as ALT >50U/L, AST >45 U/L, or GGT >55U/L; [b]total n=696

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **GGT** gamma glutamyltransferase; **HA** hyaluronic acid; **LFT** liver function test**; NPV** negative predictive value; **PPV** positive predictive value; **sens.** sensitivity; **spec.** specificity; **USS** ultrasound scan.

Table 6-3 Potential risk factors (CONTINUOUS) for unknown prevalent CS-CLD. Values are mean (sd) or median (IQR) and odds ratios (OR).

	No clinically significant CLD n=918	Prevalent clinically significant CLD (unknown) n=13	OR (95% CI)	*p*
Age, years	68.9 (4.2)	69.5 (4.9)	1.04 (0.91,1.18)	*0.600*
Glucose, mmol/L	6.87 (2.3)	7.53 (3.2)	1.11 (0.91,1.35)	*0.301*
HbA1c, %	7.19 (1.1)	7.34 (0.8)	1.13 (0.70,1.84)	*0.615*
BMI, kg/m^2	31.2 (5.6)	33.4 (6.7)	1.06 (0.98,1.16)	*0.161*
Cholesterol, mmol/L	4.2 (0.8)	3.9 (0.5)	0.68 (0.32,1.43)	*0.310*
Triglycerides, mmol/L	1.67 (0.9)	1.25 (0.3)	0.39 (0.14,1.11)	*0.078*
sBP, mmHg	138.2 (18.4)	136.9 (16.7)	1.00 (0.97,1.03)	*0.805*
CRP[b], mg/L	1.69 (0.8-3.9)	2.72 (1.4-11.6)	1.83 (1.14,2.94)	***0.013***
IL6[b], pg/ml	2.70 (1.9-4.2)	6.03 (3.1-9.6)	3.13 (1.53,6.40)	***0.002***
TNF[b], pg/ml	1.03 (0.6-1.5)	1.04 (0.9-2.5)	2.11 (1.05,4.22)	*0.035*
ALT, U/L	33.3 (12.7)	39.0 (12.6)	1.03 (1.00-1.06)	*0.108*
AST, U/L	30.0 (9.5)	49.7 (12.6)	1.09 (1.06-1.13)	***<0.001***
GGT[b], U/L	16.0 (10.0-26.0)	55.0 (34.0-103.0)	5.35 (2.91,9.83)	***<0.001***
CK18[b], U/L	100.6 (76.2-135.5)	152.7 (143.1-207.9)	3.42 (1.59,7.34)	***0.002***
APRI[b]	0.24 (0.19-0.32)	0.67 (0.35-0.92)	87.7 (20.8,369.6)	***<0.001***
AST:ALT	0.95 (0.3)	1.33 (0.3)	2.55 (1.22,5.34)	***0.013***
ELF score	8.9 (0.8)	10.9 (1.0)	4.38 (2.20,8.70)	***<0.001***
FIB4 score	1.50 (0.6)	3.96 (2.0)	7.81 (3.93,15.5)	***<0.001***
HA[b], micrg/L	50.6 (34.8-81.4)	220.3 (177.9-318.9)	16.4 (5.35,50.1)	***<0.001***
NFS	-0.40 (1.1)	1.38 (1.5)	3.99 (2.36,6.73)	***<0.001***

[a] CS-CLD defined as cirrhosis, hepatocellular carcinoma or gastro-oesophageal varices; [b] Analysed on the Ln scale: odds ratios for a one unit increase in the ln of the risk factor

ALT alanine aminotransferase; **APRI** Aspartate to Platelet Ratio Index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **CRP** C-reactive protein; **CS-CLD** clinically significant chronic liver disease; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4; **GGT** gamma glutamyltransferase; **HA** hyaluronic acid; **HR** hazard ratio; **IL6** interleukin-6; **IQR** inter-quartile range; **NFS** NAFLD Fibrosis Score; **OR** odds ratio; **sd** standard deviation; **TNFα** tumour necrosis factor-α.

Table 6-4 Potential risk factors (CATEGORICAL) for unknown prevalent clinically significant CLD. Values are % (n) and odds ratios (OR).

		No clinically significant CLD[a] n=918	Prevalent clinically significant CLD (unknown) n=13	OR (95% CI)	*p*
Sex, % male		52.5 (474)	30.8 (4)	2.49 (0.76,8.13)	*0.132*
SIMD quintile:	**I, %**	11.2 (101)	7.7 (1)	-	-
	V,%	34.9 (315)	0 (0)	-	
Duration of diabetes >5 years, %		73.7 (660)	76.9 (10)	1.19 (0.32,4.35)	*0.796*
Diabetes treatment:	**Diet, %**	19.7 (178)	15.4 (2)	Ref	-
	OAHA, %	65.1 (588)	46.2 (6)	1.06 (0.22,5.15)	*0.943*
	Insulin, %	15.2 (137)	69.2 (9)	2.60 (0.47,14.4)	*0.274*
Established hepatotoxic cause[b], %		20.7 (187)	30.8 (4)	1.70 (0.52,5.59)	*0.488*
Alcohol excess[c], %		12.6 (113)	23.1 (3)	2.09 (0.57,7.71)	*0.268*
ALT >50 U/L, %		7.8 (70)	23.1 (3)	3.56 (0.96,13.2)	*0.058*
AST >45 U/L, %		6.8 (61)	69.2 (9)	30.8 (9.22,102.9)	***<0.001***
GGT >55 U/L, %		7.9 (71)	69.2 (9)	26.2 (7.87,87.1)	***<0.001***
AST:ALT ratio >1, %		35.7 (320)	76.9 (10)	6.00 (1.64,22.0)	***0.007***
Hepatic steatosis, %		56.8 (513)	46.2 (6)	0.65 (0.22,1.95)	*0.445*
Spleen >13 cm, %		4.1 (37)	69.2 (9)	52.5 (15.5,178.5)	***<0.001***
Platelets <150x10^9/L, %		3.0 (27)	46.2 (6)	27.3 (8.59,86.6)	***<0.001***

[a] clinically significant CLD defined as cirrhosis, hepatocellular carcinoma or gastro-oesophageal varices; [b] Defined as any of: alcohol excess, hepatotoxic medication use (use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the liver assessment) or positive autoantibodies (ASMA titre >1:160 or AMA titre >1:40); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **CLD** chronic liver disease; **GGT** gamma glutamyltransferase; **HR** hazard ratio; **OR** odds ratio.

Table 6-5 Diagnostic test accuracy of pre-determined cut-offs of biomarkers for the detection of prevalent clinically significant chronic liver disease

	Unknown prevalent CS-CLD with positive measure N=13	*No CS-CLD with positive measure N=918*	*Sens., %*	*Spec., %*	*PPV, %*	*NPV, %*
CK18 >279U/L	1	52	8 (0-40)	94 (92-95)	2 (0-11)	99 (97-99)
APRI >1	3	27	23 (6-54)	97 (96-98)	10 (3-28)	99 (98-99)
ELF >10.358	11	268	85 (54-97)	71 (67-73)	4 (2-7)	99 (99-100)
FIB4 >1.30	12	516	92 (62-100)	42 (38-45)	<1 (1-4)	99 (98-100)
NFS >-1.455	12	755	92 (62-100)	14 (12-17)	2 (1-3)	99 (95-100)
NFS >0.676	10	137	77 (46-94)	84 (82-87)	7 (3-12)	99 (99-100)

APRI Aspartate to Platelet Ratio Index; **CK18** cytokeratin-18; **CS-CLD** clinically significant chronic liver disease; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4; **NFS** NAFLD Fibrosis Score; **NPV** negative predictive value; **PPV** positive predictive value; **sens.** sensitivity; **spec.** specificity.

6.4 Association of potential risk factors with incident clinically significant chronic liver disease

Following the initial liver sub-study research clinic 918 patients (Figure 6-1B) did not have prevalent clinically significant CLD.

Over a mean follow-up period of 5.6 years (sd 1.0, total 5156 person-years) there were 15 incident cases of clinically significant CLD, IR 2.9 /1000 person-years. These comprised cirrhosis n=14 (2.7 /1000 person-years), HCC n=4 (0.8 /1000 person-years), and gastro-oesophageal varices n=5 (1.0 /1000 person-years), with some patients having more than one complication. The underlying causes were attributed to: NAFLD n=12, mixed ALD/NAFLD n-1, ALD n=1 and HBV n=1.

Patients identified as potentially high risk after the liver sub-study investigations were more likely to develop clinically significant CLD than those not thought to have

liver disease (IR 10.9 vs 1.8 /1000 person-years, p=0.001). However, of the 15 patients that developed incident clinically significant CLD less than half (n=7, 46.7%) were identified as high-risk by the extensive liver-related investigations at the year 1 clinic. Of the incident HCC, only 1 (25.0%) occurred in a subject identified through the year 1 investigations. The majority of cases of varices (n=3, 60.0%) were identified through entry into an endoscopic surveillance programme after being reviewed in an NHS Hepatology clinic following the research clinic.

Rates of incident clinically significant CLD were significantly higher in those: seen in the NHS Hepatology clinic (IRR 18.7, 95%CI 6.5-54.0, p<0.001), with abnormal liver enzymes (IRR 5.7, 95%CI 2.0-16.0, p=0.001) and with very abnormal liver enzymes (IRR 16.3, 95%CI 5.2-50.9, p<0.001). Individuals with hepatic steatosis or elevated liver enzymes as defined by the lower revised laboratory reference ranges had incidence rates of clinically significant CLD not significantly different to those without (IRR 2.9, p=0.096 and IRR 1.2, p=0.800 respectively) (Table 6-6).

Patients with abnormal liver enzymes at the liver sub-study research clinic were more likely to develop incident clinically significant CLD, (normal 7/776, 0.9%; abnormal 3/113, 2.7%; very abnormal 5/26, 19.2%; p<0.001). In those developing incident clinically significant CLD, mean/median levels of liver enzymes were higher, however they remained broadly within normal laboratory limits for transaminases (ALT 44.4 U/L, AST 46.0 U/L), with median GGT levels above the upper limit of normal (median 62 vs 16 U/L, p<0.001), overall 47% of those with incident clinically significant CLD had normal liver enzymes.

Table 6-6 Incidence of developing clinically significant CLD by potential risk factors. Values are incidence rates per 1000 person-years and incidence rate ratios.

	Had potential risk factor		Did NOT have potential risk factor		IRR 95% CI	*p*
	n	IR[a]	n	IR[a]		
Seen by Hepatology services	42	30.4	876	1.6	18. 7 (6.45,54.01)	***<0.001***
Any significantly abnormal liver enzyme[b]	28	32.9	881	2.0	16.3 (5.21,50.93)	***<0.001***
Met high risk criteria following liver assessment	115	10.9	803	1.8	6.1 (2.18,17.23)	***0.001***
Any abnormal liver enzymes[c]	151	9.4	761	1.6	5.7 (2.04,16.01)	***0.001***
Hepatic steatosis	525	4.0	393	1.4	2.9 (0.82,10.50)	*0.096*
Any abnormal liver enzyme (revised criteria)[d]	701	3.0	212	2.6	1.2 (0.33,4.23)	*0.800*

[a] Incidence rate /1000 person-years; [b] Defined as any of alanine aminotransferase (ALT) >50U/L, aspartate aminotransferase (AST) >45 U/L, gamma glutamyltransferase (GGT) >55U/L; [c] Defined as any of ALT >100U/L, AST >90 U/L, GGT >110U/L; [d] Defined as any of ALT >30U/L (male), ALT>19U/L (female), AST >45 U/L, GGT >55U/L

CLD chronic liver disease; **IR** incidence rate; **IRR** incidence rate ratio.

Table 6-7 and Table 6-8 show the associations between potential risk factors and incident clinically significant CLD. There were no statistically significant differences in the age, sex or diabetes history of individuals developing clinically significant CLD compared to those who did not. The only metabolic risk factor statistically significantly associated with incident clinically significant CLD was increasing BMI (HR 1.09 p=0.016). Established hepatotoxic causes of liver disease were similar in both groups (p=0.123). Higher levels of markers of systemic inflammation were statistically significantly associated with an increased incidence of clinically significant CLD (ln CRP HR 1.66 p=0.026, ln IL-6 HR 2.86 p=0.002, ln TNF-α HR 2.12 p=0.017).

Nearly all patients (80.0%) with incident clinically significant CLD had hepatic steatosis at the start of the study, although this was not statistically significant. All of

the continuous markers of steatohepatitis and liver fibrosis (except AST:ALT ratio) were higher in those with incident clinically significant CLD than in those without (Table 6-7).

Given these associations the ‘validated’ cut-offs for NAFLD biomarkers, as described in previous chapters, were trialled as diagnostic tests alongside more commonly used clinical markers (Table 6-9). As previously the PPV of the tests remain poor resulting in large numbers of false positive results.

Table 6-7 Potential risk factors (CONTINUOUS) for incident clinically significant CLD. Values are mean (sd) or median (IQR) and hazard ratios (HR).

	No clinically significant CLD[a] n=903	Incident clinically significant CLD n=15	HR (95% CI)	*p*
Age, years	68.9 (4.2)	69.8 (4.1)	1.07 (0.94,1.21)	*0.306*
Fasting glucose, mmol/L	6.87 (2.3)	6.98 (2.8)	1.21 (0.78,1.88)	*0.405*
HbA1c, %	7.19 (1.1)	7.53 (1.5)	1.02 (0.98,1.06)	*0.405*
BMI, kg/m^2	31.2 (5.6)	34.8 (7.4)	1.09 (1.02,1.17)	*0.016*
Cholesterol, mmol/L	4.2 (0.8)	3.9 (0.8)	0.66 (0.33,1.34)	*0.248*
Triglycerides, mmol/L	1.67 (0.9)	1.46 (0.4)	0.70 (0.32,1.54)	*0.371*
sBP, mmHg	138.2 (18.4)	135.6 (21.6)	0.99 (0.96,1.02)	*0.522*
CRP[b], mg/L	1.69 (0.8-3.9)	3.98 (1.9-13.6)	1.66 (1.06,2.60)	***0.026***
IL6[b], pg/ml	2.70 (1.9-4.2)	5.31 (3.4-8.6)	2.86 (1.49,5.49)	***0.002***
TNF[b], pg/ml	1.03 (0.6-1.5)	1.45 (1.1-2.9)	2.12 (1.14,3.92)	***0.017***
ALT, U/L	33.3 (12.7)	44.4 (23.6)	1.04 (1.01,1.06)	***0.002***
AST, U/L	30.0 (9.5)	46.0 (23.7)	1.07 (1.04,1.09)	***<0.001***
GGT[b], U/L	16.0 (10.0-26.0)	62.0 (21.5-185.0)	3.56 (2.17,5.83)	***<0.001***
CK18[b], U/L	100.6 (76.2-135.5)	127.6 (83.4-586.5)	4.10 (2.08,8.06)	***<0.001***
APRI[b]	0.24 (0.19-0.32)	0.39 (0.29-0.88)	20.4 (6.81,61.0)	***<0.001***
AST:ALT	0.95 (0.3)	1.10 (0.3)	1.56 (0.93,2.64)	*0.094*
ELF score	8.9 (0.8)	10.2 (1.0)	1.64 (1.30,2.06)	***<0.001***
FIB4 score	1.50 (0.6)	2.56 (1.3)	4.08 (2.71,6.15)	***<0.001***
HA[b], micrg/L	50.6 (34.8-81.4)	183.2 (78.9-230.6)	5.80 (2.60,13.0)	***<0.001***
NFS	-0.40 (1.1)	0.74 (1.0)	2.18 (1.54,3.09)	***<0.001***

[a] clinically significant CLD defined as cirrhosis, hepatocellular carcinoma or gastro-oesophageal varices; [b] Analysed on the Ln scale: hazard ratios for a one unit increase in the ln of the risk factor.
ALT alanine aminotransferase; **APRI** Aspartate to Platelet Ratio Index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **CLD** chronic liver disease; **CRP** C-reactive protein; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4; **GGT** gamma glutamyltransferase; **HA** hyaluronic acid; **HR** hazard ratio; **IL6** interleukin-6; **IQR** inter-quartile range; **NFS** NAFLD Fibrosis Score; **OR** odds ratio; **sd** standard deviation; **TNFα** tumour necrosis factor-α.

Table 6-8 Potential risk factors (CATEGORICAL) for incident clinically significant CLD. Values are % (n) and hazard ratios (95%CI).

		No clinically significant CLD[a] n=903	Incident clinically significant CLD n=15	HR (95% CI)	*p*
Sex, % male		52.5 (474)	40.0 (6)	1.93 (0.69,5.45)	*0.213*
SIMD quintile:	**I, %**	11.2 (101)	33.3 (5)	-	
	V,%	34.9 (315)	26.7 (4)	0.26 (0.07,0.97)	*0.044*
Duration of diabetes >5 years, %		73.7 (660)	93.3 (14)	5.30 (0.70,40.34)	***0.107***
Diabetes treatment:	**Diet, %**	19.7 (178)	6.7 (1)	-	
	OAHA, %	65.1 (588)	53.3 (8)	2.50 (0.31,20.0)	*0.388*
	Insulin, %	15.2 (137)	40.0 (6)	9.08 (1.09,75.5)	***0.041***
Established hepatotoxic cause[b], %		20.7 (187)	40.0 (6)	2.44 (0.87,6.85)	*0.091*
Alcohol excess[c], %		12.6 (113)	20.0 (3)	1.63 (0.46,5.77)	*0.451*
ALT >50 U/L, %		7.8 (70)	40.0 (6)	6.69 (2.38,18.8)	***<0.001***
AST >45 U/L, %		6.8 (61)	40.0 (6)	8.30 (2.94,23.43)	***<0.001***
GGT >55 U/L, %		7.9 (71)	53.3 (8)	13.2 (4.79,36.6)	***<0.001***
AST:ALT ratio >1, %		35.7 (320)	60.0 (9)	3.67 (1.29,10.44)	***0.015***
Hepatic steatosis, %		56.8 (513)	80.0 (12)	2.93 (0.83,10.38)	*0.096*
Spleen >13 cm, %		4.1 (37)	0	-	-
Platelets <150x10^9/L, %		3.0 (27)	14.3 (2)	4.36 (0.97,19.6)	*0.055*

[a] clinically significant CLD defined as cirrhosis, hepatocellular carcinoma or gastro-oesophageal varices; [b] Defined as any of: alcohol excess, hepatotoxic medication use (use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the liver assessment) or positive autoantibodies (ASMA titre >1:160 or AMA titre >1:40); [c] Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **CLD** chronic liver disease; **GGT** gamma glutamyltransferase; **HR** hazard ratio; **OR** odds ratio.

Table 6-9 Diagnostic test accuracy of pre-determined cut-offs of biomarkers for the detection of incident clinically significant chronic liver disease

	Incident CS-CLD with positive measure N=903	*No CS-CLD with positive measure N=15*	*Sens. %*	*Spec., %*	*PPV, %*	*NPV, %*
Any abnormal LFT[a]	9	152	60 (33-83)	83 (81-86)	6 (3-11)	99 (98-100)
CK18 >279U/L	4	49	31 (10-61)	94 (92-96)	8 (2-19)	99 (98-99)
HA >100	13	336	87 (58-98)	64 (61-67)	4 (2-7)	99 (99-100)
APRI >1.0	2	28	20 (5-49)	97 (96-98)	11 (3-29)	99 (98-99)
AST:ALT >1	9	328	50 (27-74)	64 (61-67)	3 (1-5)	99 (97-99)
ELF >10.358	10	269	67 (39-87)	71 (68-74)	4 (2-7)	99 (98-100)
FIB4 >1.30	13	515	92 (64-100)	42 (39-46)	3 (1-4)	100 (98-100)
NFS >-1.455	13	754	93 (64-100)	14 (12-17)	2 (1-3)	99 (95-100)
NFS >0.676	8	139	57 (30-81)	84 (82-87)	5 (3-11)	99 (98-100)

[a] Defined as ALT >50U/L, AST >45 U/L, or GGT >55U/L

ALT alanine aminotransferase; **APRI** Aspartate to Platelet Ratio Index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4; **HA** hyaluronic acid; **LFT** liver function test; **NFS** NAFLD Fibrosis Score.

6.5 Overall burden of clinically significant chronic liver disease

By the end of the follow-up period, 36 patients were identified as having either new or existing clinically significant CLD. This included eight known prevalent cases (all cirrhosis), 13 unknown prevalent cases diagnosed as a result of the liver sub-study investigations (12 cirrhosis only and 1 cirrhosis and HCC) and 15 incident cases during the follow-up period (7 with cirrhosis only, 2 cirrhosis and HCC, 4 cirrhosis and varices, 1 cirrhosis, HCC and varices and 1 HCC only). Of the 36 patients, in total there were 35 (3.7%) cases of cirrhosis, 9 (1.0%) HCC cases, and 11 (1.2%) cases of gastro-oesophageal varices. Of note there was 1 HCC occurring in a patient without cirrhosis.

Potential risk factor associations with the total burden of clinically significant CLD are shown in Table 6-7 and Table 6-8. Findings were similar to those for incident clinically significant CLD, with the addition of statistical association with surrogate markers of portal hypertension (platelet count $<150x10^9$/L and spleen diameter >13cm)

Table 6-10 Potential risk factors (CONTINUOUS) for ever developing clinically significant CLD. Values are odds ratios (95%CI).

	OR (95% CI)	*p*
Age, years	1.06(0.98,1.15)	*0.133*
Fasting glucose, mmol/L	1.04 (0.91,1.20)	*0.545*
HbA1c, %	1.00 (0.73,1.37)	*0.984*
BMI, kg/m^2	1.06 (1.01,1.11)	***0.020***
Cholesterol, mmol/L	0.66 (0.42,1.04)	*0.073*
Triglycerides, mmol/L	0.57 (0.33,1.00)	*0.051*
sBP, mmHg	0.99 (0.97,1.01)	*0.254*
CRP[a], mg/L	1.75 (1.32,2.32)	***<0.001***
IL6[a], pg/ml	2.64 (1.72,4.04)	***<0.001***
TNF[a], pg/ml	2.30 (1.55,3.41)	***<0.001***
ALT, U/L	1.03 (1.01,1.05)	***0.001***
AST, U/L	1.07 (1.05,1.08)	***<0.001***
GGT[a], U/L	3.84 (2.84,5.20)	***<0.001***
CK18[a], U/L	3.50 (2.25,5.45)	***<0.001***
APRI[a]	19.44 (10.83,34.88)	***<0.001***
AST:ALT	1.75 (1.34,2.28)	***<0.001***
ELF score	1.66 (1.45,1.91)	***<0.001***
FIB4 score	2.30 (1.98,2.68)	***<0.001***
HA[a], micrg/L	6.11 (3.67,10.15)	***<0.001***
NFS	2.43 (1.97,2.99)	***<0.001***

[a] Analysed on the Ln scale: odds ratios for a one unit increase in the ln of the risk factor
ALT alanine aminotransferase; **APRI** Aspartate to Platelet Ratio Index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **CRP** C-reactive protein; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4; **GGT** gamma glutamyltransferase; **HA** hyaluronic acid; **IL6** interleukin-6; **IQR** inter-quartile range; **NFS** NAFLD Fibrosis Score; **OR** odds ratio; **sd** standard deviation; **TNFα** tumour necrosis factor-α.

Table 6-11 Potential risk factors (CATEGORICAL) for ever developing clinically significant CLD. Values are odds ratios (95% CI).

		OR (95% CI)	*p*
Sex, % male		1.96 (1.00,3.84)	*0.050*
SIMD quintile:	**I, %**	-	**-**
	V,%	0.29 (0.10,0.79)	***0.016***
Duration of diabetes >5 years, %		1.86 (0.77,4.47)	*0.186*
Diabetes treatment:	**Diet, %**	-	-
	OAHA, %	1.60 (0.55,4.66)	*0.388*
	Insulin, %	4.00 (1.27,12.58)	***0.018***
Established hepatotoxic cause[a], %		2.85 (1.48,5.51)	***0.002***
Alcohol excess[b], %		2.16 (1.01,4.59)	***0.046***
ALT >50 U/L, %		4.29 (2.11,8.73)	***<0.001***
AST >45 U/L, %		12.49 (6.48,24.08)	***<0.001***
GGT >55 U/L, %		16.11 (8.23,31.51)	***<0.001***
AST:ALT ratio >1, %		4.57 (2.27,9.21)	***<0.001***
Hepatic steatosis, %		0.93 (0.48,1.79)	*0.819*
Spleen >13 cm, %		7.83 (3.76,16.31)	***<0.001***
Platelets <150x10^9/L, %		10.61 (5.19,21.72)	***<0.001***

[a]Defined as any of: alcohol excess, hepatotoxic medication use (use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the liver assessment) or positive autoantibodies (ASMA titre >1:160 or AMA titre >1:40); [b]Defined as females >14 units/week, males >21 unis/week or patient disclosed history of a current or prior alcohol problem.

ALT alanine aminotransferase; **AST** aspartate aminotransferase; **GGT** gamma glutamyltransferase; **OR** odds ratio.

6.6 Summary

In this chapter, I found that the prevalence of clinically significant CLD (defined as cirrhosis, HCC or gastro-oesophageal varices) was 2.2% - 0.8% diagnosed prior to enrolment with an additional 1.4% identified by study investigations. Over nearly 6 years of follow-up, only 1.4% of the cohort developed incident clinically significant CLD.

Higher levels of systemic inflammation, steatohepatitis and hepatic fibrosis markers were associated with both unknown prevalent and incident clinically significant chronic liver disease (all $p<0.001$). Less than half of participants developing incident significant disease were identified as high risk by the study investigations. Abnormal liver enzymes were associated with incident cases (IRR 5.7, $p=0.001$), the presence of hepatic steatosis was not. Several markers had good sensitivity for the identification of CS-CLD but poor PPV (<10%) meant high numbers of false positives also occur.

CHAPTER 7 Results III: Association between liver markers and cardiovascular events

Patients with chronic liver disease, and in particular NAFL/D, are known to have both higher all-cause mortality[171,172] and CV mortality rates than the general population[113,173]. Although links between liver disease and CVD have frequently been attributed to the inter-relationships between NAFL/D, obesity and the metabolic syndrome, it has emerged that there is an increased risk of CV events in subjects with NAFLD, independent of these traditional risk factors[188,189,346,347].

To date, the most commonly cited hepatic marker of CV mortality[193,198] and incident CVD[186,187] is plasma GGT. However, reports are conflicting with some refuting the association[173,192] and others finding attenuation with increasing age[200]. It is not clear if the relationship relates to a specific hepatic influence on CVD or whether GGT is a surrogate marker for systemic inflammation.

Despite extensive studies of GGT there has been little investigation of the relationship between CVD and other markers of liver injury (non-specific liver injury, steatosis, steatohepatitis, liver fibrosis or portal hypertension).

In this chapter I have:

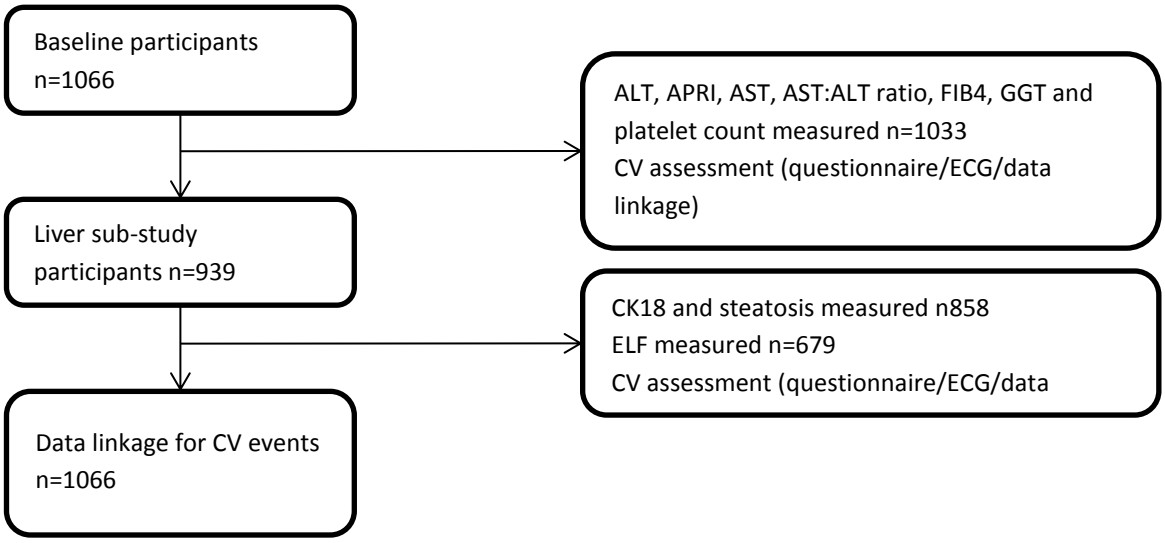
(i) described the association of a range of liver markers with both prevalent and incident CV events and mortality. Liver markers include: non-specific liver injury (ALT, AST and GGT), steatosis, steatohepatitis (CK18), liver fibrosis (APRI, ELF, FIB4, NFS), and surrogates of portal hypertension (platelet count)

7.1 Study population

In order to maximise the follow-up time available for incident CVD events to occur where possible baseline liver markers were used (ALT, APRI, AST, AST:ALT ratio, FIB4, GGT and platelet count), with the year 1 liver sub-study clinic used where data

was unavailable (steatosis, CK18 and ELF). Figure 7-1 shows the subject flow and data availability for the analyses in this chapter. ELF data was available on a limited random subgroup of n=679 subjects (missing data discussed previously Section 5.1.2).

Figure 7-1 Flowchart of cardiovascular outcomes in the Edinburgh Type 2 Diabetes Study



ALT alanine aminotransferase; **AST** aspartate aminotransferase; baseline baseline; **CK18** cytokeratin-18; **CV** cardiovascular; **ECG** electro-cardio graph; **GGT** gamma glutamyltransferase; liver sub-study liver study;

7.2 Association of liver markers with cardiovascular events

There were 372/1033 (36.0%) patients with prevalent CVD and 319/1033 (30.9%) with prevalent CAD at baseline. A significantly higher proportion of those with CVD and CAD were male (both 61.8%, p<0.001). Those with CVD and CAD were older (mean 68.4 vs 67.6 years, p=0.004, and 68.6 vs 67.6 years, p<0.001, respectively) than those without. Full results of the associations between established risk factors for CVD and prevalent CVD are shown in Table 7-1. Results were similar for the 1 year assessment with 303/858 (35.3%) patients with prevalent CVD and 260/858 (30.3%) with prevalent CAD at baseline. Again those with CVD and CAD were significantly more likely to be male and to be older.

Table 7-1 Association of established cardiovascular risk factors with prevalent clinical cardiovascular events in all patients, values are mean (sd), median (IQR) or % (n).

		Prevalent CVD, n=370	No CVD, n=663	*p*	Prevalent CAD, n=317	No CAD, n=716	*p*
Age, years		68.4 (4.4)	67.8 (4.1)	***0.005***	68.6 (4.4)	67.6 (4.1)	***0.001***
Sex, % male		61.9 (229)	45.2 (300)	***<0.001***	61.8 (196)	46.5 (333)	***<0.001***
SIMD:	**I** **V**	15.9 (59) 25.7 (95)	10.0 (66) 36.8 (244)	***<0.001***	16.7 (53) 25.9 (82)	10.1 (72) 35.9 (257)	***<0.001***
Duration of diabetes,		7.0 (4.0-11.3)	6.0 (3.0-10.0)	***0.001***	7.0 (4.0-11.0)	6.0 (3.0-11.0)	***0.007***
HbA1c, %		7.42 (1.1)	7.38 (1.1)	*0.532*	7.41 (1.1)	7.38 (1.1)	*0.707*
Diabetes treatment:	**Diet** **OAHA** **Insulin**	17.8 (63) 60.6 (214) 21.5 (76)	20.9 (134) 64.2 (412) 15.0 (96)	***0.027***	17.6 (53) 61.5 (185) 20.9 (63)	20.7 (144) 63.5 (411) 15.7 (109)	*0.105*
BMI, kg/m^2		31.6 (5.4)	31.1 (5.7)	*0.190*	31.6 (5.4)	31.2 (5.7)	*0.253*
sBP, mmHg		132.8 (17.4)	133.5 (15.8)	*0.543*	133.0 (17.8)	133.3 (15.8)	*0.798*
dBP, mmHg		68.1 (9.5)	69.7 (8.7)	***0.008***	67.9 (9.6)	69.6 (8.7)	***0.003***
Lipid lowering therapy, % yes		92.4 (342)	81.8 (541)	***<0.001***	93.4 (296)	82.2 (587)	***<0.001***
BP lowering therapy, %yes		90.8 (335)	77.6 (510)	***<0.001***	91.8 (290)	78.2 (555)	***<0.001***
HDL cholesterol,		1.20 (0.3)	1.35 (0.4)	***<0.001***	1.20 (0.3)	1.34 (0.4)	***<0.001***
Total cholesterol,		4.21 (0.9)	4.35 (0.9)	***0.013***	4.18 (0.9)	4.35 (0.9)	***0.004***
Smoking: % ever		68.2 (202)	56.8 (325)	***0.001***	67.9 (169)	57.8 (358)	***0.007***
Alcohol excess, % yes		9.5 (35)	7.4 (49)	*0.285*	9.1 (29)	7.7 (55)	*0.459*
eGFR,		62.1 (16.0)	65.7 (13.9)	***<0.001***	61.5 (15.9)	65.7 (14.0)	***<0.001***

BMI body mass index; **BP** blood pressure; **CAD** coronary artery disease; **CVD** cardiovascular disease; **dBP** diastolic BP; **eGFR** estimated glomerular filtration rate; **HDL** high density lipoprotein; **OAHA** oral antihyperglycaemic agent; **sBP** systolic BP; **SIMD** Scottish Index of Multiple Deprivation.

Table 7-2 shows the mean/median values of each of the markers of liver injury in those with and without prevalent CV events. Mean ALT was marginally lower in those with prevalent CVD (41.9 vs 43.7 U/L, p=0.048). GGT values were statistically significantly higher than in those with both prevalent CVD and CAD (median 20.0 vs 17.0 U/L, p<0.001, for both) (p<0.001) – although all median levels were within the normal range. Surprisingly, whilst just missing statistical significance, the presence of hepatic steatosis was more frequently found in the absence of CV events (CVD 54.1 vs 57.5% and CAD 51.2 vs 58.5%).

Multivariable analysis adjusting for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation, smoking status, excess alcohol consumption, BMI, systolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and eGFR is shown in Table 7 3 and Table 7 4. GGT was the only liver marker independently statistically associated with prevalent CVD (OR for a doubling of GGT 1.18, p=0.021) and CAD (OR 1.21, p=0.008).

Table 7-1 Association of markers of liver injury with prevalent clinical cardiovascular events in all patients, values are mean (sd), median (IQR) or % (n).

	CVD, yes	CVD, no	*p*	CAD, yes	CAD, no	*p*
ALT, U/L	41.9 (12.6)	43.7 (14.2)	***0.048***	42.1 (13.0)	43.5 (14.0)	*0.164*
AST, U/L	30.4 (10.5)	31.3 (10.0)	*0.178*	30.5 (11.0)	31.2 (9.8)	*0.282*
GGT, U/L	20.0 (13-37)	17.0 (10-29)	***<0.001***	20.0 (13-37)	17.0 (11-29)	***<0.001***
Steatosis, % yes	54.1 (164)	57.5 (319)	*0.350*	51.2 (133)	58.5 (350)	*0.051*
CK18, U/L	100.7 (79-141)	104.8 (77-137)	*0.911*	99.1 (74-138)	105.8 (78-138)	*0.589*
APRI	0.25 (0.19-0.35)	0.25 (0.20-0.33)	*0.848*	0.25 (0.19-0.36)	0.25 (0.20-0.33)	*0.860*
AST/ALT ratio	0.74 (0.2)	0.73 (0.2)	*0.759*	0.74 (0.2)	0.74 (0.2)	*0.869*
ELF score	8.9 (0.8)	8.9 (0.9)	*0.930*	8.9 (0.8)	8.9 (0.9)	*0.950*
FIB4	1.39 (0.7)	1.34 (0.6)	*0.164*	1.42 (0.7)	1.33 (0.6)	*0.060*
NFS	-27.0 (2.5)	-27.4(2.6)	***0.018***	-26.9 (2.5)	-27.4 (2.6)	***0.010***
Platelets, x10^9/L	254.5 (72.9)	260.1 (67.8)	*0.240*	252.4 (74.3)	260.8 (67.4)	*0.094*

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase, **CAD** coronary artery disease; **CK18** cytokeratin-18; **CVD** cardiovascular disease; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

Table 7-2 Multivariable association between liver markers and all prevalent cardiovascular events. Values are odds ratios (95%CI)

	Model 1	*p*	Model 2	*p*	Model 3	*p*
ALT, U/L	0.990 (0.979,1.001)	*0.079*	0.987 (0.976,0.999)	***0.028***	0.989 (0.977,1.002)	*0.088*
AST, U/L	0.993 (0.979,1.007)	*0.341*	0.990 (0.975,1.005)	*0.174*	0.993 (0.977,1.009)	*0.385*
GGT, U/L[1]	1.211 (1.073,1.366)	***0.002***	1.195 (1.057,1.352)	***0.005***	1.178 (1.025,1.355)	***0.021***
Steatosis, % yes	0.907 (0.676,1.218)	*0.518*	0.957 (0.708,1.298)	*0.774*	0.837 (0.599,1.169)	*0.296*
CK18, U/L[1]	1.080 (0.895,1.304)	*0.421*	1.085 (0.896,1.313)	*0.405*	0.990 (0.805,1.218)	*0.926*
APRI[1]	0.976 (0.778,1.225)	*0.833*	0.852 (0.670,1.082)	*0.189*	0.904 (0.701,1.67)	*0.439*
AST:ALT ratio	1.342 (0.561,3.212)	*0.509*	1.392 (0.564,3.435)	*0.473*	1.507 (0.558,4.074)	*0.419*
ELF score	1.002 (0.828,1.213)	*0.984*	1.005 (0.818,1.234)	*0.964*	0.940 (0.744,1.188)	*0.604*
FIB4	1.128 (0.903,1.410)	*0.289*	1.012 (0.799,1.282)	*0.921*	1.033 (0.801,1.332)	*0.801*
NFS	1.043 (0.986,1.103)	*0.144*	1.063 (1.003,1.127)	***0.038***	1.043 (0.971,1.120)	*0.248*
Platelets, x10^9/L	0.999 (0.997,1.001)	*0.569*	1.001 (0.998,1.003)	*0.499*	1.000 (0.998,1.002)	*0.734*

[1] APRI, CK18 and GGT analysed on the Log_2 scale for linearization, therefore odds ratios relate to a doubling of the marker.

Model 1 - Unadjusted

Model 2 - Adjusted for age and sex

Model 3 - Adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, body mass index, systolic blood pressure, diastolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and estimated glomerular filtration rate.

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

Table 7-3 Multivariable association between liver markers and prevalent coronary artery events. Values are odds ratios (95%CI)

	Model 1	*p*	Model 2	*p*	Model 3	*p*
ALT, U/L	0.993 (0.981,1.004)	*0.200*	0.991 (0.976,1.003)	*0.124*	0.994 (0.982,1.007)	*0.390*
AST, U/L	0.993 (0.978,1.008)	*0.383*	0.991 (0.975,1.006)	*0.230*	0.995 (0.979,1.012)	*0.588*
GGT, U/L[1]	1.223 (1.080,1.385)	***0.002***	1.218 (1.073,1.384)	***0.002***	1.213 (1.051,1.401)	***0.008***
Steatosis, % yes	0.747 (0.549,1.017)	*0.064*	0.789 (0.577,1.080)	*0.140*	0.661 (0.462,0.935)	***0.019***
CK18, U/L[1]	1.047 (0.860,1.274)	*0.650*	1.053 (0.864,1.282)	*0.610*	0.959 (0.777,1.184)	*0.707*
APRI[1]	0.998 (0.788,1.265)	*0.987*	0.881 (0.687,1.131)	*0.320*	0.953 (0.733,1.239)	*0.720*
AST:ALT ratio	1.011 (0.404,2.532)	*0.981*	0.933 (0.360,2.416)	*0.887*	0.943 (0.334,2.661)	*0.912*
ELF score	0.980 (0.801,1.200)	*0.848*	0.961 (0.772,1.194)	*0.726*	0.881 (0.685,1.133)	*0.324*
FIB4	1.197 (0.952,1.504)	*0.123*	1.067 (0.838,1.359)	*0.599*	1.107 (0.854,1.434)	*0.441*
NFS	1.044 (0.985,1.107)	*0.146*	1.061 (0.999,1.127)	*0.055*	1.045 (0.971,1.125)	*0.237*
Platelets, x10^9/L	0.999 (0.997,1.001)	*0.386*	1.000 (0.998,1.002)	*0.788*	1.000 (0.997,1.002)	*0.967*

[1] APRI, CK18 and GGT analysed on the Log_2 scale for linearization, therefore odds ratios relate to a doubling of the marker.

Model 1 - Unadjusted

Model 2 - Adjusted for age and sex

Model 3 - Adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, body mass index, systolic blood pressure, diastolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and estimated glomerular filtration rate. Incident analysis additionally adjusted for prevalence cardiovascular disease at baseline.

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

7.3 Association of liver markers with incident cardiovascular events

There were 663 participants without CVD. After a mean follow-up of 4.4 years from baseline attendance there were 44/663 (6.6%) patients with incident CVD and 27/663 (4.1%) with incident CAD events. A significantly higher proportion of those with incident CVD were male (59.1% vs 44.3%, p=0.061) and they were significantly older (68.9 vs 67.5 years, p=0.024), with no differences in those with incident CAD compared with those without incident CAD. Similar results were obtained for those patients followed up from the 1 year assessment (mean follow-up 3.5 years), with 35/561 (6.2%) incident CVD and 19/561 (3.4%) incident CAD events and with a similar age/sex distribution. Full results of the associations between established CV risk factors and incident CV events are shown in Table 7-4.

There were 82/1,033 (7.9%) deaths in the follow-up period from baseline, with 30/82 (36.6%) attributable to CVD, of which 20 were attributable to CAD.

Mean (or median) liver injury marker levels were largely similar between participants with and without incident CVD (Table 7-5) and after multivariable adjustment (Table 7-6 and Table 7-7). Only GGT appeared to have some independent association with either incident CVD (HR for a doubling of GGT 1.24; 95% CI 0.97, 1.59; p=0.086) or incident CAD (HR 1.33; 95% CI 1.00, 1.78; p=0.053). None of the individual co-variables added to the multivariable model had a major attenuating effect on the HR estimating the GGT–outcome association. The addition of inflammatory variables to the model did result in attenuation of the HR point estimate (Table 7-8) for the GGT-outcome association. With regards to different oral anti-hyperglycaemic agents only the use of TZDs attenuated the GGT-outcome association (Table 7-9).

Table 7-4 Association of established cardiovascular risk factors with incident cardiovascular events in all patients, values are mean (sd), median (IQR) or % (n).

		Incident CVD, n=44	No incident CVD, n=619	*p*	Incident CAD, n=27	No CAD, n=636	*p*
Age, years		68.9 (3.7)	67.5 (4.1)	***0.024***	68.8 (4.1)	67.5 (4.1)	*0.121*
Sex, % male		59.1 (26)	40.9 (18)	*0.061*	51.9 (14)	48.1 (13)	*0.555*
SIMD:	**I**	18.2 (8)	9.4 (58)	*0.232*	18.5 (5)	9.6 (61)	*0.590*
	V	31.8 (14)	37.2 (230)		33.3 (9)	36.9 (235)	
Duration of diabetes, years		7.0 (4.0-12.8)	6.0 (3.0-10.0)	*0.088*	7.0 (4.0-12.0)	6.0 (3.0-10.0)	*0.248*
Diabetes treatment:	**Diet**	17.1 (7)	21.1 (127)		19.2 (5)	20.9 (129)	
	OAHA	53.7 (22)	64.9 (390)	***0.029***	46.2 (12)	64.9 (400)	***0.015***
	Insulin	29.3 (12)	14.0 (84)		34.6 (9)	14.1 (87)	
BMI, kg/m^2		31.1 (4.8)	31.1 (5.7)	*0.981*	31.0 (5.1)	31.1 (5.7)	*0.899*
sBP, mmHg		137.6 (14.1)	133.2 (15.9)	*0.069*	135.8 (13.2)	133.3 (15.9)	*0.436*
dBP, mmHg		70.5 (7.2)	69.6 (8.8)	*0.523*	68.6 (6.6)	69.7 (8.8)	*0.505*
Lipid lowering therapy, % yes		86.4 (38)	81.5 (503)	*0.545*	81.5 (22)	81.9 (519)	*1.000*
BP lowering therapy, %yes		81.8 (36)	77.3 (474)	*0.577*	85.2 (23)	77.3 (487)	*0.479*
HDL cholesterol,		1.26 (0.3)	1.35 (0.4)	*0.092*	1.30 (0.4)	1.35 (0.4)	*0.534*
Total cholesterol,		4.33 (1.0)	4.35 (0.9)	*0.889*	4.50 (1.1)	4.35 (0.9)	*0.354*
Smoking: % ever smoked		34.2 (13)	43.8 (234)	*0.310*	52.0 (13)	57.0 (312)	*0.682*
Alcohol excess, % yes		11.4 (5)	7.1 (44)	*0.363*	7..4 (2)	7.4 (47)	*1.000*
eGFR, ml/L		63.0 (14.3)	65.9 (13.8)	*0.196*	66.1 (14.0)	65.7 (13.9)	*0.867*

BMI body mass index; **BP** blood pressure; **CAD** coronary artery disease; **CK18** cytokeratin-18; **CVD** cardiovascular disease; **dBP** diastolic BP; **eGFR** estimated glomerular filtration rate; **HDL** high density lipoprotein; **OAHA** oral antihyperglycaemic agent; **sBP** systolic BP; **SIMD** Scottish Index of Multiple Deprivation.

Table 7-5 Association of markers of liver injury with incident clinical cardiovascular events in patients without pre-existing cardiovascular disease, values are mean (sd), median (IQR) or % (n).

	CVD, yes *N=35*	*CVD, no* *N=628*	*p value*	*CAD, yes* *N=19*	*CAD, no* *N=644*	*p value*
ALT, U/L	46.0 (16.7)	43.5 (14.1)	0.266	48.2 (19.1)	43.5 (13.5)	0.218
AST, U/L	34.2 (13.0)	31.1 (9.7)	0.047	35.6 (14.6)	31.1 (9.7)	0.125
GGT, U/L	21.0 (10-37)	16.0 (10-27)	0.102	19.0 (9-56)	17.0 (10-28)	0.504
Steatosis, % yes[1]	50.0 (15)	57.8 (307)	0.450	43.8 (7)	57.8 (315)	0.309
CK18, U/L[1]	108.9 (85-146)	103.6 (76-137)	0.356	102.1 (84-176)	104.8 (77-137)	0.724
APRI	0.26 (0.21-0.34)	0.25 (0.20-0.33)	0.669	0.26 (0.20-0.40)	0.25 (0.20-0.33)	0.782
AST/ALT ratio	0.75 (0.2)	0.73 (0.2)	0.357	0.75 (0.1)	0.73 (0.2)	0.640
ELF score[2]	9.3 (0.8)	8.9 (0.9)	0.066	9.1 (0.7)	8.9 (0.9)	0.416
FIB4	1.39 (0.6)	1.33 (0.6)	0.494	1.42 (0.6)	1.33 (0.6)	0.417
NFS	-0.81 (1.1)	-0.72 (1.1)	0.600	-0.90 (1.0)	-0.72 (1.1)	0.440
Platelets, x10^9/L	266.9 (65.6)	259.8 (69.7)	0.502	269.7 (68.4)	259.9 (67.8)	0.460

Mean follow-up 4.4 years, except for CK18, ELF and steatosis where mean follow-up was 3.5 years.

1 Incident CVD n=30/561 incident CAD n=16/561; 2 incident CVD n=24/444 incident CAD n=13/444.

ALT alanine aminotransferase; APRI aspartate aminotransferase to platelet ratio index; AST aspartate aminotransferase, CAD coronary artery disease; CK18 cytokeratin-18; CVD cardiovascular disease; ELF Enhanced Liver Fibrosis; FIB4 Fibrosis-4 score; GGT gammaglutamyl transferase; NFS NAFLD Fibrosis Score

Table 7-6 Multivariable association between liver markers and any incident cardiovascular disease events. Values are hazard ratios (95%CI)

Liver marker	*Model 1*	*p value*	*Model 2*	*p value*	*Model 3*	*p value*
ALT, U/l	1.00 (0.97, 1.02)	0.754	1.00 (0.97, 1.02)	0.836	0.99 (0.97, 1.02)	0.669
AST, U/l	1.01 (0.98, 1.04)	0.526	1.01 (0.98, 1.04)	0.544	1.01 (0.97, 1.04)	0.700
GGT, log_2[1]	1.25 (0.99, 1.59)	0.062	1.26 (0.99, 1.60)	0.059	1.24 (0.97, 1.59)	0.086
Steatosis, % yes	0.78 (0.36, 1.67)	0.525	0.84 (0.39, 1.80)	0.654	0.90 (0.40, 2.00)	0.787
CK18, log_2[1]	1.05 (0.64, 1.70)	0.857	1.13 (0.68, 1.85)	0.643	1.02 (0.60, 1.75)	0.931
APRI, log_2[1]	0.88 (0.505, 1.525)	0.644	0.79 (0.43, 1.46)	0.448	0.76 (0.40, 1.45)	0.408
AST:ALT ratio	3.63 (0.61, 21.61)	0.156	2.85 (0.475, 17.06)	0.252	3.58 (0.53, 28.12)	0.183
ELF score	1.220 (0.91, 1.64)	0.185	1.19 (0.85, 1.66)	0.312	1.15 (0.81, 1.64)	0.443
FIB4	1.01 (0.54, 1.91)	0.966	0.82 (0.40, 1.68)	0.586	0.83 (0.39, 1.76)	0.625
NFS	0.81 (0.58, 1.14)	0.226	0.76 (0.54, 1.06)	0.109	0.78 (0.57, 1.09)	0.143
Platelets, $\times 10^9$/l	1.00 (1.00, 1.01)	0.162	1.01 (1.00, 1.01)	0.061	1.00 (1.00, 1.01)	0.110

[1] APRI, CK18 and GGT analysed on the Log_2 scale for linearization, therefore odds ratios relate to a doubling of the marker.

Model 1 - Unadjusted

Model 2 - Adjusted for age and sex

Model 3 - Adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, body mass index, systolic blood pressure, diastolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and estimated glomerular filtration rate. Incident analysis additionally adjusted for prevalence cardiovascular disease at baseline.

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

Table 7-7 Multivariable association between liver markers and incident coronary artery disease events. Values are hazard ratios (95%CI)

Liver marker	*Model 1*	*p value*	*Model 2*	*p value*	*Model 3*	*p value*
ALT, U/l	1.00 (0.98, 1.03)	0.771	1.01 (0.98, 1.04)	0.497	1.01 (0.98, 1.04)	0.611
AST, U/l	1.02 (0.99, 1.05)	0.213	1.03 (0.99, 1.06)	0.135	1.02 (0.99, 1.06)	0.220
GGT, log_2[a]	1.27 (0..95, 1.69)	0.103	1.31 (9.88, 1.75)	0.060	1.33 (1.00, 1.78)	0.053
Steatosis, % yes	0.82 (0.32, 2.14)	0.688	0.87 (0.33, 2.27)	0.774	0.91 (0.33, 2.53)	0.858
CK18, log_2[a]	1.07 (0.58, 1.99)	0.822	1.10 (0.60, 2.01)	0.748	0.96 (0.49, 1.90)	0.908
APRI, log_2[a]	1.07 (0.56, 2.06)	0.839	1.15 (0.56, 2.34)	0.709	1.10 (0.52, 2.32)	0.804
AST:ALT ratio	4.36 (0.51, 37.18)	0.178	3.40 (0.37, 31.13)	0.278	4.25 (0.39, 46.73)	0.237
ELF score	1.24 (0.85, 1.80)	0.269	1.15 (0.76, 1.74)	0.508	1.12 (0.69, 1.82)	0.642
FIB4	1.28 (0.64, 2.60)	0.486	1.22 (0.57, 2.64)	0.611	1.25 (0.56, 2.79)	0.583
NFS	0.84 (0.55, 1.28)	0.416	0.81 (0.53, 1.23)	0.323	0.76 (0.51, 1.17)	0.225
Platelets, $\times 10^9$/l	1.00 (1.00, 1.01)	0.301	1.00 (1.00, 1.01)	0.286	1.00 (1.00, 1.01)	0.297

[1] APRI, CK18 and GGT analysed on the Log_2 scale for linearization, therefore odds ratios relate to a doubling of the marker.

Model 1 - Unadjusted

Model 2 - Adjusted for age and sex

Model 3 - Adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, body mass index, systolic blood pressure, diastolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and estimated glomerular filtration rate. Incident analysis additionally adjusted for prevalence cardiovascular disease at baseline.

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

Table 7-8 Multivariable association between liver markers and incident cardiovascular disease events, effects of inflammatory markers. Values are hazard ratios (95%CI)

Liver marker	*CVD*	*p value*	*CAD*	*p value*
GGT, log_2	1.26 (1.03, 1.55)	*0.027*	1.27 (0.95, 1.69)	*0.103*
+ age and sex	1.27 (1.03, 1.56)	*0.027*	1.30 (1.00, 1.69)	*0.050*
+ cardiovascular risk factors[1]	1.21 (0.94, 1.55)	*0.134*	1.26 (0.94, 1.67)	*0.118*
+ inflammatory markers[2]	1.23 (1.00, 1.52)	*0.054*	1.28 (0.98, 1.66)	*0.066*
+age and sex and cardiovascular risk factors	1.24 (0.97, 1.59)	*0.086*	1.33 (1.00, 1.78)	*0.053*
+ all of above	1.16 (0.89, 1.52)	*0.275*	1.34 (0.98, 1.85)	*0.071*

1 defined as duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, BMI, systolic blood pressure (sBP), diastolic blood pressure (dBP), HbA1c, HDL cholesterol, total cholesterol and eGFR. ; **2** defined as c-reactive protein, interleukin-6 and tumour necrosis factor alpha

CAD coronary artery disease; **CVD** cardiovascular disease; **GGT** gamma-glutamyltransferase

Table 7-9 Multivariable association between liver markers and incident cardiovascular disease events, effects of different oral anti-hyperglcaemic agents. Values are hazard ratios (95%CI)

Liver marker	*CVD*	*p value*	*CAD*	*p value*
GGT, log_2	1.26 (1.03, 1.55)	*0.027*	1.27 (0.95, 1.69)	*0.103*
+ metformin	1.26 (1.00, 1.60)	*0.054*	1.25 (0.91,1.70)	*0.163*
+ sulphonylurea	1.26 (1.00, 1.60)	*0.054*	1.25 (0.91, 1.70)	*0.164*
+ glitazone	1.21 (0.96, 1.53)	*0.109*	1.20 (0.88, 1.64)	*0.250*

CAD coronary artery disease; **CVD** cardiovascular disease; **GGT** gamma-glutamyltransferase

In further analyses performed on all participants with either a first or subsequent CV event occurring after baseline (i.e. including those with prevalent CVD at baseline, but with adjustment for prevalent cases), an association between GGT and events was confirmed (Appendix M). HRs with similar magnitudes were observed with increased statistical significance ($p<0.05$), likely due to the increase in sample size.

When restricted to patients with NAFLD (n=319) there were 38 incident cardiovascular events, with 23 attributable to CAD. Of all the liver injury markers

investigated, GGT alone showed an independent association with incident CVD in this subgroup (fully adjusted HR for a doubling of GGT 1.56; 95% CI 1.08, 2.28; p=0.019) (Appendix M)

7.4 Summary

In this chapter, I found that at baseline there were 372/1035 patients with prevalent CVD, including 319/1035 with CAD. After mean follow-up of 4.4 years there were 44/663 incident CVD events, including 27/663 CAD events. There were 30/82 CVD related deaths.

I was able to replicate existing knowledge of the relationship between GGT and CVD and in addition I was able to add that this finding persisted into older age and that other liver markers (steatosis, steatohepatitis, liver fibrosis and surrogates of portal hypertension) were not associated with CV events.

CHAPTER 8 Discussion

8.1 Overview

This thesis aimed to investigate the epidemiology of CLD in older people with type 2 diabetes. In this chapter I discuss the main findings of this work in the context of previous literature. Additionally, I discuss key strengths and weakness of the thesis and finish with potential directions for future research.

8.2 Key findings of thesis

8.2.1 Non-invasive markers of steatohepatitis and liver fibrosis in older people with type 2 diabetes

In this community based cohort of older people with type 2 diabetes, examination of a wide range of potential markers of steatohepatitis and liver fibrosis found varied relationships with diabetes history. AST:ALT ratio, ELF, FIB4, and LSM were statistically associated with hyperglycaemia, and ELF and HA with increasing duration of diabetes. Elevated ELF was associated with OAHA and insulin use and APRI, FIB4 and NFS with diet control. Most commonly, elevated markers of steatohepatitis and liver fibrosis were associated with older age and higher body fat measures (BMI and waist circumference). However, most of these relationships between liver markers and body fat measures lost statistical significance when limiting the population to only those with hepatic steatosis and/or NAFL/D. The presumption that poorer diabetes control (indicated by higher HbA1c) would be related to elevated steatohepatitis and fibrosis markers was not proven.

As noted above, there were differences in the associations between different liver fibrosis markers and potential diabetes and metabolic risk factors, suggesting that these markers are not actually measuring the same underlying "fibrosis" condition. There was poor correlation between the five markers of liver fibrosis studied. Using

the top vigintile (5%) of each marker resulted in excellent agreement on the absence of advanced liver disease but poor agreement on the presence of advanced liver disease.

8.2.2 Clinically significant chronic liver disease in older people with type 2 diabetes – prevalence, incidence and associated risk factors

The prevalence of clinically significant CLD (defined as cirrhosis, HCC or gastro-oesophageal varices) was 2.2% - 0.9% diagnosed prior to enrolment with an additional 1.4% identified by study investigations. Over nearly 6 years of follow-up, only 1.4% of the cohort developed incident clinically significant CLD.

Higher levels of systemic inflammation, steatohepatitis and hepatic fibrosis markers were associated with both unknown prevalent and incident clinically significant chronic liver disease (all $p<0.001$). Less than half of participants developing incident significant disease were identified as high risk by the study investigations. Abnormal liver enzymes were associated with incident cases (IRR 5.7, $p=0.001$), the presence of hepatic steatosis was not.

8.2.3 Association between liver markers and cardiovascular events

At baseline there were 372/1035 patients with prevalent CVD, including 319/1035 with CAD. After mean follow-up of 4.4 years there were 121/1035 incident CVD events, including 76/1035 CAD events. There were 30/82 CVD related deaths.

However, risk of dying from or developing CVD was no higher in subjects with steatosis than without (OR 0.84, $p>0.05$). There was also no statistically significant relationship between CVD and steatohepatitis or liver fibrosis. The only statistically significant relationship between CVD and any liver markers was with GGT (prevalent CVD, OR 1.28, $p=0.007$; incident CAD, OR 2.35, $p=0.042$), suggesting that in our study population, CLD may have little effect on the development of, or mortality from, CVD.

8.3 Study strengths and limitations

8.3.1 Study population

A major strength of this thesis is that it provides a comprehensive assessment of a wide range of markers of steatohepatitis and liver fibrosis in a population-based cohort of all older people with type 2 diabetes, and not just subjects selected primarily on the diagnosis of hepatosteatosis using recruitment from diabetes clinics at tertiary referral centres as in previous studies[161,162,167,348]. I have provided diabetes-specific information on the distribution of these markers, which is essential to inform further research on the clinical relevance of possible subclinical liver dysfunction in this high risk group.

The sample was drawn from the whole of the Lothian region, and included the full spectrum of people with type 2 diabetes. Whilst in general the study population was largely representative of the target population of older people with type 2 diabetes there were some differences. These differences (e.g. sex) were small at baseline, but sometimes statistically significant most likely because of the large sample size. I also only examined representativeness in a limited number of variables chosen because of a) their availability, and b) their presumed influence on outcomes of interest. It may be that there were other variables not measured which differed and had an influence on the results. This would in turn influence the generalisability of the results. However, overall the ET2DS can be considered generalisable to the target population (high external validity).

Retention of nearly 80% (831/1066) of the cohort between the baseline and follow-up clinics is good. Differences in attenders and non-attenders at the year 1 liver sub-study clinic and year 4 follow-up are probably in the expected direction: non-attenders were slightly older with higher systolic BP and from more deprived areas, which likely reflects higher morbidity and mortality. These differences are inevitable when relying on clinic attendance in an elderly population. Again the differences are small, but could have reached statistical significance if sample size was larger. The effects of this attrition were mitigated to some extent by the use of

other means of follow-up such as GP questionnaires and data linkage with hospital discharge data.

At both baseline and follow-up the power calculations were undertaken with respect to cognitive function outcomes. For example, a follow-up sample size of 800 (allowing for a combined 20% death and drop-out rate), was deemed to be powered at 90% to detect a correlation coefficient of 0.12 and above between change in cognitive test scores and each of the risk factors. There were no a priori liver sub-study power calculations undertaken as the ET2DS cohort had already been recruited prior to its commencement. Given that the sample is large, it is likely that the analyses undertaken were powered sufficiently to detect similar differences in the cross-sectional analyses of liver markers. However, the analyses of both prevalent and incident clinically significant CLD may be underpowered.

Given that there were lower prevalences of high levels of non-invasive markers (Chapter 5) and less cases of clinically significant CLD than perhaps anticipated (Chapter 6) it has to be questioned whether the ET2DS study population is perhaps unusual. It is evident from the representativeness analysis that the study subjects are representative of all older people with type 2 diabetes in Scotland, however it may be that older people with type 2 diabetes show some sort of healthy survivor bias i.e. they are biologically different to those not surviving to age 60 years. A brief comparison of mortality in the ET2DS compared to the age-sex matched Lothian and Scottish figures (Appendix N) suggests that this is not the case with the mortality rate in the ET2DS exceeding that of Lothian and Scotland (25 vs 16 vs 17 per 1000 population/year respectively).

8.3.2 Missing data

Some variables (e.g. BMI, platelets) were only measured at the baseline clinic and not at the initial liver sub-study visit. As a result, for some analyses these measures are used interchangeably. This is a reasonable decision to take as these are all variables that would not be expected to change significantly over such a short time period.

Another noteable limitation is the volume of missing data relating to LSM (15%). The XL probe is believed to increase the success rates of obtaining ≥10 valid TE readings from 56% to 75%[292] in obese populations. This is consistent with the 85% success in a mixed overweight and obese cohort. Despite the use of the XL probe, TE still appears to be limited by body habitus. Those patients with missing LSM had more severe diabetes profiles and higher alternative markers of liver fibrosis. Hence, it was felt necessary to expand the majority of analysis to those patients with a full set of markers excluding LSM in order to reduce biasing the sample. However, interpretation of the results of any analyses relating to LSM need to be undertaken with the recognition that it is not generalizable to all older people with diabetes.

8.3.3 Study design

This thesis is subject to the inherent disadvantages of an observational cohort study. Many of the analyses relate to cross-sectional studies where it is not possible to determine the order of events ie which came first the elevated liver marker or the elevated metabolic risk factor? Cross-sectional design also relies on CLD having a long natural history, as any sort duration illnesses would be under represented by such a design.

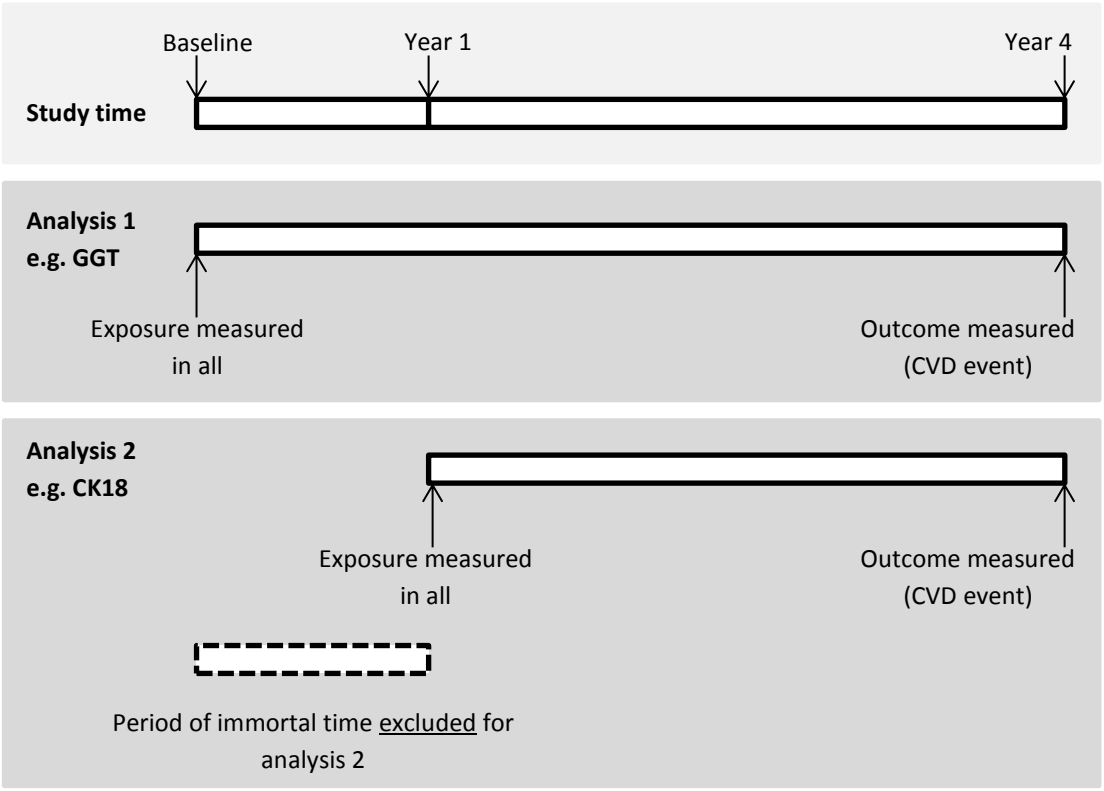
The prospective design of the ET2DS allowed the investigation of the relationship between baseline risk factors and subsequent clinical outcomes (clinically significant CLD and CVD). The natural history of CLD is long. By collating cases of clinically significant CLD beyond the follow-up research clinic we were able to extend the follow-up period for incident cases to nearly six years (Chapter 6). Despite this, six years is not long in the liver disease pathway.

8.3.4 Immortal time bias

Immortal person-time arises in an observational study when follow-up time is included in person-time at risk for the study outcome, even though that time precedes the exposure definition[349]. An exposure category that includes immortal person-time leads to a downwardly biased outcome rate and an upwardly biased survival curve.

This bias occurs because the accumulated person-time exceeds person-time actually at risk. Particular consideration was given to this issue when analysing incident CVD events in Chapter 7. Here, some exposures were measured at the baseline research clinic and others at the year 1 liver sub-study visit, allowing for the potential for immortal time to arise. The two most widely advocated ways to deal with the issue of immortal person-time is a) to only compare this patients surviving the immortal time period or b) allow for time varying covariates in the analyses. Because the different exposure variables were treated as different analyses in Chapter 7, immortal time was excluded from the analyses (see Figure 8-1). The exposure (e.g. CK18 testing) occurred to all individuals at the same time and there was therefore no difference between the 'exposed' and 'unexposed' groups. A consequence of this action is potential selection and survival bias, as discussed previously. If in analysis 2 of the example below (Figure 8-1) patient time had been in included prior to the exposure and all of these attributed to the 'non-exposure' group, immortal time misclassification would have occurred.

Figure 8-1 Immortal time bias example



CK18 cytokeratin-18; **CVD** cardiovascular disease; **GGT** gamma-glutamyltransferase

8.3.5 Data quality

All research staff were appropriately trained in the collection of data and standard operating procedures were followed to reduce observer bias. Training in the use of TUE (Fibroscan, Echosens) was undertaken by the external company and the single assessor had to reach an acceptable standard set by Echosens prior to commencement of the study.

All Edinburgh based laboratory assays were measured in the same laboratory at all study phases, reducing any risk of biases from changes in measurement protocols on the results.

Both CK18 and ELF were measured at external laboratories in Nottingham and London respectively. All CK18 samples were assayed as a minimum in duplicate and where sample volumes allowed they were measured up to four times with the median result taken as the final value. The coefficient of variation for the high control sample was 3.3% (mean 694u/l) and for the low control 7.1% (mean 104u/l). The iQur laboratory in London reported for the ELF measurements that "the results have been validated and passed quality assurance".

All analysed measurements of LSM met the quality guidance issued by the developer (at least 60% of 10 LSM successful, and the inter-quartile range <30% of the final, median, result). A number of measures did not meet this criteria so were not used. Use of a single TUE operative eliminated the risk of any inter-observer variability, however, I was unable to arrange for any repeated measures of LSM on individuals to be undertaken to allow the intra-observer variation to be assessed.

8.3.6 Number of analyses

The number of analyses undertaken and p values reported in this thesis is large (approximately 1500). As discussed in section 4.7.4 one potential approach to the resulting high risk of Type I statistical error is to apply Bonferroni adjustment to p-values. As described this type of adjustment beings its own difficulties so was not used in this thesis. Had it been applied across the whole of the thesis the Bonferroni-adjusted

p-value indicating statistical significance would be $p<0.00003$, and (presumably) few or none of the analyses would survive this adjustment. However, the overall pattern of results suggests that a majority of findings with $p<0.05$ reflect more than chance results, so that the findings presented here likely to be relatively accurate reflections of the described associations. Furthermore, where-ever possible confidence intervals around point estimates have been included so that context specific decisions about the statistical versus clinical significance can be made.

As noted previously as significant proportion of this thesis involved the generation of hypotheses as opposed to targeted hypothesis testing. Significant marker findings could now be taken forward and for example validated in a new cohort.

8.3.7 Agreement statistics

There are a number of ways of assessing agreement between markers and to assess the (inter-observer) agreement of liver fibrosis markers I reported proportions of positive and negative agreement (sections 4.7.3 and 5.8). A common alternative approach would be to use the Kappa statistic. The Kappa statistic assesses the difference between how much agreement is actually present ("observed" agreement) compared to how much agreement would be expected to be present by chance alone ("expected" agreement)[350]. A problem with the Kappa statistic is that it is affected by the prevalence of the disease under consideration - for uncommon findings, very low values of kappa can result which may not reflect actual high rates of overall agreement[342,343,350]. To avoid this issue the alternative approach of separating positive and negative agreement was used.

8.4 Non-invasive markers of steatohepatitis and liver fibrosis in older people with type 2 diabetes

8.4.1 Distributions of markers of steatohepatitis and liver fibrosis

In Chapter 5, I demonstrated the distributions of CK18, AST:ALT ratio, APRI, ELF, FIB4, NFS and LSM in older people with type 2 diabetes. In terms of the

distributions of potential steatohepatitis and fibrosis markers, in both populations with diabetes and community based cohorts, there is limited evidence available in individuals unselected for liver disease.

My findings for the distribution of CK18 in people with type 2 diabetes were consistent with the 'general population' assay literature[351]. In developing the normal ranges for the serum CK18 assay, 200 healthy Swedish blood donors were tested; as in my study, the results showed similar levels in males and females with little change in levels with increasing age and an overall marker distribution similar to the one I found. In a second study [351], a normal cut-point of the 80th percentile, or 145U/L, was suggested, and this is also consistent with our finding (146U/L).

For ELF, Yoo et al suggest a normal range of 5.95-8.73 in apparently healthy South Korean subjects[352]. I found that ELF scores were very slightly lower in men and increased with age. In the absence of a biologically plausible reason to expect any difference in any of the components of ELF by sex, it is possible that the higher ELF scores in females may truly represent more advanced liver fibrosis. The components of ELF (HA, TIMP-1 and P3NP) are all related to extra-cellular matrix turnover and are not exclusive to the liver. As a result, one might expect an increase in ELF with age, both due to the greater time in which liver fibrosis has had to develop[107] and due to increasing prevalence of unrelated causes of raised analytes; indeed, consistent with my own findings, an early study examining HA and P3NP found higher levels in 'healthy' elderly people compared with younger subjects [353] .

I found LSM ranged from 0.37-33.30 kPa (mean 5.07 kPa), with similar levels in males and females. There have been several 'community-based' studies of TUE. In France, Roulot et al[354] offered all individuals aged >45 years who attended for a scheduled medical check-up (no additional selection information provided) the opportunity to be screening for CLD with TUE. 1190 individuals participated and the range of LSM was 1.8-35kPa, median 5.3kPa. These results were similar to my own, however, they found significantly higher levels in males than females (5.7 versus 4.9kPa). Based on a randomly selected general population cohort in Hong Kong,

Wong et al suggest that the normal range of LSM in people without fatty liver or CLD is 2.8-7.4kPa[355].

8.4.2 Associations with potential risk factors

I determined a number of relationships between potential risk factors (diabetes history and metabolic factors) and non-invasive markers of steatohepatitis and hepatic fibrosis. However, these relationships were typically small with correlation coefficients <0.2. A challenge in interpreting these results clinically is the lack of validated marker cut-points to diagnose hepatic inflammation and/or fibrosis in population-based cohorts. Despite this, these results suggest that at least a number of metabolic risk factors (including fasting glycaemia, BMI and waist circumference) are likely to be associated with liver fibrosis and/or inflammation in people with type 2 diabetes.

In terms of diabetic populations, it is not known whether the measures and scores of non-invasive markers of steatohepatitis (CK18) and liver fibrosis (APRI, AST:ALT ratio, ELF, FIB4, NFS and LSM) investigated differ, on average, from non-diabetic populations, although there is also no known biological reason why this should be the case. For this reason, alongside concerns over the use of validated cut-offs, I chose the top quintile of the marker distribution as the highest risk groups. Whilst the imprecision of such an approach in terms of diagnosing disease must be acknowledged, I have shown that the highest quintiles of each of the markers contained higher surrogate markers of portal hypertension, providing some confirmatory evidence that this group included a particularly high risk group of patients in terms of clinically significant CLD. In addition I was able to confirm that the presence of other conditions known to influence levels of the markers (such as chronic kidney disease and arthritis) do not appear to have a major effect on the results (i.e. unlikely to confound the results).

The finding of associations of hepatic steatosis, increased body fat, hyperglycaemia and more intensive diabetes treatment with steatohepatitis and liver fibrosis markers may appear to support the hypothesis that poor diabetes control and a worse

metabolic profile are involved in increasing the risk of developing CLD. However, these findings were not consistent across markers. In a recent liver biopsy study [348] in people with type 2 diabetes, in which high rates of both NASH (78%) and moderate fibrosis (34-60%) were detected, no statistical associations between diabetes related/metabolic factors and NASH or liver fibrosis were found. However, this biopsy study was small (n=98) and focused on patients at the severe end of the diabetes spectrum attending a tertiary referral hospital.

The cross-sectional nature of these analyses associating liver markers of steatohepatitis and liver fibrosis with diabetes and metabolic risk factors limits any temporal inference; it is not possible to determine whether metabolic factors might be a risk factor for liver disease or vice versa. However, if causal relationships were to be confirmed, this would have important implications for strategies aimed at CLD risk reduction e.g. losing or redistributing fat and reducing insulin resistance. Although associations between the markers and metabolic factors appeared relatively weak, addressing even weak risk factors for disease could be beneficial at a population level, especially if those risk factors are highly prevalent.

In addition to the association of steatohepatitis and fibrosis markers with metabolic risk factors, I was also interested in their association with steatosis and established hepatotoxic causes (alcohol excess, hepatotoxic medication use and strongly positive autoantibodies) of CLD. I found that subjects with hepatic steatosis had higher CK18 and LSM measures and lower AST:ALT ratios and HA measures compared with non-steatotic individuals, but not APRI, ELF FIB4 or NFS. This is perhaps unsurprising given that CK18 levels rise with increasing hepatic inflammation as a by-product of hepatocellular apoptosis and that according to established models of NAFL/D progression[62,356], initial development of hepatic steatosis is followed by NASH and then hepatic scarring with steatosis typically receding as fibrosis progresses.

I again found varied associations between established hepatotoxic causes and any steatohepatitis or liver fibrosis markers, with some markers being higher in those with established hepatotoxic causes (APRI, FIB4, LSM and NFS), some being lower

(CK18, ELF and HA), and AST:ALT ratio showing no association. These varied results may be explained, at least in part, by the small numbers of study subjects with high levels of the established hepatotoxic causes (alcohol excess 11.8%, hepatotoxic medication use 3.6%, strongly positive autoantibodies 0.6%) and by lack of consensus around the precise level of risk factor which should be used to establish increased risk. I defined alcohol excess using cut-points which are consistent with the published literature in the UK and I was unable to find any consensus in the literature on how best to define hepatotoxic medications in terms of what types, duration and dosage are required to have a significant effect on the liver[61].

One of the most consistent findings in the current study was the association of all the investigated markers of steatohepatitis and liver fibrosis with measures of increasing body fat (except HA). Previous studies have shown a direct association between liver fat and hepatic inflammation, with the latter increasing proportionately according to liver fat volume[357]. It has been proposed that this effect is mediated though the direct release of toxic free fatty acid by hepatic fat and through altered lipid partitioning within hepatocytes, mitochondrial dysregulation, generation of reactive oxygen species, lipid peroxidation and endoplasmic reticulum stress[358]. Given the relationship between visceral fat and inflammation, the finding of increased body fat in patients in the highest CK18 quintile is consistent with proposed underlying mechanisms of hepatic inflammation[63,359]. In addition to increased body fat, subjects in the top CK18 quintile also had higher fasting glucose and plasma HbA1c levels, as well as more intensive diabetes treatment modalities. These factors may be considered as surrogates of beta cell failure and worsening insulin resistance, which is in turn related to hepatic inflammation through increased lipolysis, increased free fatty acid presence in the liver and ultimately oxidative stress[360]. As in other studies [361,362], I found higher triglyceride levels with increased CK18 levels, which would be consistent with the theory of free fatty acids driving lipid accumulation in the form of triglycerides in the liver in NAFL.

8.4.3 Marker agreement

Overall there was poor correlation between the fibrosis markers measured and compared in this study. The only exception was APRI and FIB4 (r=0.92 in full cohort and 0.93 in NAFL/D subgroup, p<0.001), which is unsurprising given they have a number of components in common (AST level and platelets). Assessment of inter-item correlation (α=0.67) determined that the five markers were not consistent with each other in what they were measuring, with a score of α=0.70 being the minimum score usually accepted. However, a minimum of 0.90 is often suggested for clinical practice[363]. This suggests some discrepancy in what the markers are measuring. This seems plausible as AST/ALT, APRI and FIB4 are all similar in their composition, including markers of hepatocellular damage. Conversely, ELF is measuring markers related to extracellular matrix turnover in fibrosis, and LSM is examining structural properties of the liver through shear wave transmission.

A higher prevalence of fibrosis in the NAFL/D cohort compared to the full cohort was expected. However, using validated cut-offs, my results showed the opposite. This probably reflects the natural history of NAFL/D in which fibrotic progression is often associated with steatosis regression and hence this group of advanced liver disease patients might not be captured by my definition of NAFL/D. ELF scores and LSM values for the top vigintile were most in keeping with the values one would expect to find from previous published studies[159,161,163], however, with no reference standard (biopsy) it is impossible to establish which of the five markers is the most accurate and to comment on the true prevalence of liver fibrosis using non-invasive markers.

In addition, using validated cut-offs for advanced fibrosis, I found a wide range of fibrosis prevalence results for the different markers (0.8-68.3%). The reasons for the wide discrepancies are probably two-fold. Firstly, as demonstrated by the lack of inter-correlation, there is an inconsistency in what the different markers/panels are measuring. Secondly, published fibrosis cut-offs from clinical validation studies do not appear to translate readily into research or clinical practice. The difficulty with this is that the predictive value of a test (unlike sensitivity and specificity) is

influenced by the prevalence of underlying disease. Most validation cohorts have typically comprised a high proportion of patients with advanced liver disease selected from tertiary referral centres. The study population used in this thesis has a presumed lower prevalence of advanced liver disease and consequently the predictive values are likely to be different. This resulting lower positive predictive value and higher negative predictive value would mean that literature based cut-offs are neither reliable nor directly comparable with one another in different patient cohorts. Without liver biopsy (the current reference standard) it is not possible to decide which marker is 'best suited' for diagnosing significant liver fibrosis in a lower prevalence population.

There are numerous other markers of liver fibrosis available as discussed in Chapter 2 e.g. BARD, BAAT, NFS. These are typically simple scoring systems using easily available plasma results and patient data. Previous work from the ET2DS investigators has found that these scores are likely to overestimate the prevalence of liver fibrosis in populations similar to mine as they rely heavily on the incorporation of impaired glucose tolerance, age and body mass index. For example the prevalence of fibrosis using the BARD and BAAT scores was 92.6% and 79.3% respectively, with the NFS predicting 16.4% fibrosis and 66.8% indeterminate[364]. It is therefore necessary to concentrate on the development of liver fibrosis markers that are independent of the underlying characteristics of the population under study.

A further area which requires clarification is the most appropriate use of hepatic markers in the general/healthy population. Without this information, it is hard to predict whether expected values are likely to differ in discrete populations, such as the elderly or people with diabetes. Normal routine liver function tests vary with age, sex and ethnicity[329,365], and therefore any fibrosis marker panel including these components might benefit from specific reference ranges that reflect the individual population.

8.5 Clinically significant chronic liver disease in older people with type 2 diabetes – prevalence, incidence and associated risk factors

In Chapter 6, I reported that the prevalence of clinically significant CLD was 2.2% - 0.9% diagnosed prior to study enrolment with an additional 1.4% identified by study investigations. Over nearly 6 years of follow-up, only 1.4% of the cohort developed incident clinically significant CLD.

The existing literature on prevalence and incidence of clinically significant CLD is limited by its heavy reliance on confirmatory liver biopsy in secondary care populations or, in community-based cohorts, the use of a single screening modality (e.g. FibroTest[242], transient elastography[354]). Single 'disease' classifiers each bring their own limitations, for example liver enzymes have low diagnostic accuracy, non-invasive fibrosis markers (e.g. ELF, NFS) have poor positive predictive values for later stages of CLD[236,243], ICD codes only identify hospitalised cases with decompensation, and biopsy has marked ascertainment bias. A study by Wong et al highlighted the challenge of applying such markers in a general population, finding the prevalence of advanced fibrosis in NAFL/D to range from 0-12% depending on the markers used[355]. This methodological variation, coupled with the high 'unknown' prevalence of clinically significant CLD, provides an important explanation for discordance in the literature regarding prevalence rates of clinically significant CLD. Another issue to note with identifying CLD is that it is predominantly an out-patient treated condition, therefore not easily identified from hospital discharge (SMR01) records. Therefore, I used a combination of methods to comprehensively phenotype our cases including radiology, clinical codes, non-invasive markers and clinician verification. Each of these methods has their own limitations, and reviewing all medical records is highly time consuming and not feasible for larger studies, but together they provide a detailed phenotype within the current limitations of diagnostic tools and lends confidence in the ascertainment of clinically significant CLD given the multimodal approach. It would have been preferable to have been able to include some level of advanced fibrosis in the clinically significant CLD definition however this was not possible because it was

rarely coded or referred to in medical records, most likely due to the need for liver biopsy to confidently diagnose it.

My findings support the suggestion that NAFL/D is an under-diagnosed chronic disease[366] despite patients with type 2 diabetes having, as a minimum, annual clinical reviews including liver enzyme tests. The extensive study investigations increased the prevalence by more than 150% through the diagnosis of unknown clinically significant CLD, of which almost 70% was attributable to NAFL/D. However, it was a labour intensive process with 14% of the study subjects referred to a Consultant Hepatologist. In the majority of these patients, simple laboratory tests were abnormal (e.g. liver enzymes above the upper limit of normal, low platelet count) although, typically, values were only marginally elevated.

Reassuringly, despite a relatively high prevalence of uncomplicated NAFL/D at year 1 of the study, there were only a small number of patients who went on to develop incident clinically significant CLD after 6 years of follow-up (2.9 /1000 person-years). Adams et al[109] followed 103 NAFL/D patients over a mean period of 3.2 years and found a fibrosis progression rate of 0.35 stages/year in those with diabetes. This is high compared with the ET2DS cohort as it equates to a 2-stage advancement over the 6 year follow-up of the cohort in those with NAFL/D. Whilst difficult to quantify, given a NAFL/D prevalence in the cohort of 43%[165] with 60% of incident clinically significant CLD being attributable to NAFL/D, this would indicate that around 2% of the non-cirrhotic NAFL/D subjects had F2-3 disease at the start of the study, with nearly 98% having F0-1. In NASH populations, Harrison et al[116] found that 32% of their cohort progressed fibrosis score over 3 years and, similarly, Fassio et al[115] showed that 31% of subjects had progressive fibrosis although no subject developed cirrhosis during 4 years of follow-up.

A further difficulty in the interpretation of NAFL/D prevalence/incidence is the diagnostic criteria for NAFL/D. In this thesis a criteria in line with many others was taken – presence of steatosis on USS, absence of secondary cause including, positive autoantibodies, hepatotoxic medication use and excess alcohol consumption. Increasingly incidence data is being driven by evaluation of large linked datasets as

opposed t using well phenotyped cohorts as in this study. As a result diagnoses are a) often limited to secondary care only, and b) the exclusion criteria are often poorly recorded. Further to that, what exclusion criteria so use can vary by study. The majority of incident cases in my work were attributed to NAFL/D. The measurement of exclusion criteria was robust – perhaps with the exception of alcohol. This is related to two issues: the difficulties there are measuring alcohol intake and knowing at what level to determine NAFL/D from ALD.

Various methodological issues influence the measurement of alcohol consumption in research. Firstly, the period of time information is recalled for – a short recent period vs a longer time to allow for a recognition that there may be variation in drinking patterns, but issues of actual recall. Two commonly used measurement approaches are the usual quantity/frequency and graduated frequency approaches, both of which allow researchers to estimate the volume of alcohol intake. Other issues include whether to ask drink type specific questions and estimation of drink sizes. Finally the mode of collection, face-to-face vs "anonymous" influences the responders behaviour[367-369]. In this thesis I used a quantity/frequency approach, asking face-to-face over the past year. This may have resulted in under reporting of alcohol consumption. The cut-off criteria then applied were the 'safe-drinking' limits advised by the Royal College of Physicians[70]. Some argue that they should be higher[61], however, this stricter criteria helps to offset any patient under reporting.

I did not find any aspect of diabetes history to be associated with the development of clinically significant CLD. However, it is notable that my incidence rate was 3.6 times higher than that found by Porepa et al [370] in a large population-based study in Canada using similar non-biopsy related outcomes to our own, where those with newly diagnosed diabetes had an incidence rate of 0.82 /1000 person-years (twice that of those without diabetes) and I had over 90% of my incident cases occurring in subjects with a diabetes duration of >5 years. A recent systematic review of cross-sectional studies found the presence of diabetes to significantly increase the risk of HCC in both case-control and cohort studies with a pooled OR 2.5 (95% CI 1.8-3.5) and a pooled RR 2.5 (95% CI 1.9-3.2)[371]. In addition, Scottish Cancer Registry data

shows that within the whole Scottish population aged 60-75 the IR for liver-related cancer (including biliary) in 2012 was 0.3/1000 person-years[20]. This is substantially lower than the rate for HCC alone in the ET2DS cohort (0.8/1000 person-years). In a prospective study, El-Serag et al found the incidence of HCC in patients with diabetes to be 0.2/1000 person-years, 2.7 times the rate of their control group[372]. This indicates that the presence of diabetes is associated with increased cirrhosis and HCC risk.

I found that incident clinically significant CLD was associated with higher measures of body fat, markers of systemic inflammation and a wide range of markers of liver disease. Indeed, all three markers of systemic inflammation were associated with the development of clinically significant CLD. The role of inflammatory mediators in the development of cirrhosis and HCC due to virtually any underlying CLD has been noted previously[373].

Critically, the majority of people with incident clinically significant CLD had normal liver function tests with no steatosis and so were not identified by the extensive research clinic assessment. Whilst those with abnormal liver enzymes *per se* were more likely to develop clinically significant CLD, the mean levels of liver enzymes in those that did were still within the normal laboratory reference range. The lack of statistical significance between hepatic steatosis and the development of clinically significant CLD may be due to the small number of outcomes or it may be a true. If true, it could reflect that steatosis and NAFL confers little additional risk over that already present in people with type 2 diabetes due to their shared background of insulin resistance.

It is not surprising that markers of liver fibrosis were associated with the future development of clinically significant CLD given they have been developed to diagnose that disease process. Previously work from the ET2DS group found the NFS to be problematic for diagnosing liver fibrosis in a population with type 2 diabetes due to its composition leading to an over estimation of prevalence[364]. However, the NFS may be of benefit in the prediction of future liver related events. In a large general population cohort, McLernon at al[374] created a prediction model

for incident CLD using routinely collected data, although this had a PPV of just 40% and only examined short term incidence (up to 2 years).

Given the associations found between the various markers of liver injury with both prevalent and incident clinically significant CLD I trialled the use of pre-determined cut-offs to diagnose clinically significant CLD. Whilst Chapter 5 determined that 'validated' cut-offs of markers for hepatic fibrosis could not be used for the diagnosis of fibrosis, it may have been that they could be used for this alternative purpose – prediction of future disease. Despite good sensitivity of some tests (>85%) all of the corresponding PPVs were below 10% meaning that in clinical practice large numbers of patients would have false positive results. This would mean a large burden of work for clinical services and also subjecting a lot of patients unnecessarily to further investigation. Combining the most sensitive of the routine markers (AST. ALT, GGT, platelet count, spleen size) still did not produce a test with sufficient positive predictive power to result in a clinically acceptable test for screening for liver disease.

Receiver operator curves were not constructed to determine the optimal cut-off point for markers of liver injury in the diagnosis of clinically significant CLD as the number of outcomes (n=15) was too small to have the power to provide accurate results with confidence.

Despite the findings of this work, it is still not clear how 'screening' programmes for clinically significant CLD could be structured. Beyond trying to identify subsets of the population who might benefit most from further investigation the practical aspects of the process require consideration.

Because the different non-invasive markers of liver disease are all potential useful, in addition to their clinical utility factors such as access and cost need to be considered. For example, routine liver enzymes (ALT, AST and GGT) can be measured within all existing hospital laboratory services at an approximate cost of £0.50, £1.00 and £0.50. Serum HA is a more specialist test, available in most hospital laboratories, at a cost of £8.00-10.00 per sample. ELF is a commercially available test currently only

processed UK at the iQur Laborarotory in London at a standard cost of approximately £80 per test.

8.6 Association between liver markers and cardiovascular events

In Chapter 7, I found a statistically significant association between GGT and both prevalent and incident CV events, but no association between other liver markers investigated (AST, ALT, steatosis, CK18, APRI, AST:ALT ratio, ELF, FIB4, NFS and platelet count) and CVD.

My findings are consistent with previous reports that GGT is independently associated with both prevalent and incident CV events and that this association is not attenuated by increased age. In addition, my work supports CAD as the main element of association within the spectrum of CVD. Perhaps surprisingly, no other markers of liver injury - hepatic steatosis, inflammation or fibrosis - were associated with CV events.

This work consolidates previous findings of an association between GGT and both prevalent and incident CVD, with the largest effect on CAD[162,375-379]. There is biological plausibility for this relationship. GGT degrades glutathione to glutamate, which via cysteinylglycine is involved in iron reduction, allowing lipoprotein oxidation within atheromatous plaques. What is unclear is whether GGT is a pathogenic factor in atherogenesis or simply a surrogate marker of the micro-inflammatory plaque-associated inflammatory response.

The relationship between transaminases and CVD is controversial, with reports of significant associations in both directions[347,380,381]. A number of centres have previously investigated the relationship between NAFL/D (presence of hepatic steatosis on USS) and CV events[184,382], with sample populations comprised exclusively of patients with type 2 diabetes[162,189,346,376]. In this study, there was no relationship between transaminases or sonographic hepatic steatosis and CVD.

CK18 is an apoptosis marker released by injured hepatocytes. It has been demonstrated that in patients with NAFLD, relative concentrations can differentiate between steatosis and NASH[84]. There are no previous studies examining the relationship between CK18 and CV events. Several prior studies diagnosing NASH using different methods (biopsy, elevated ALT levels) showed mixed results for the association of CV risk (e.g. risk scores, lipid levels)[383-385]. Both Soderberg[183] and Ekstedt[113] found associations between all-cause and CV mortality with the presence of biopsy proven NASH, but no association with steatosis. Conversely Lazo[386] found no association NASH and CV mortality in patients diagnosed by USS and elevated hepatic enzymes – suggesting that the criteria and methodology for NAFL/D and NASH classification may have a significant impact in determining prognostic value.

Data on the relationship between liver fibrosis and CVD is also limited. Kim et al found significant associations between the NAFLD Fibrosis Score, aspartate to platelet ratio index and the FIB-4 Score with CV mortality[387]. Our study used the ELF score, an extracellular matrix related multi-component panel (HA, P3NP and TIMP-1), validated for use in patients with NAFL/D[236] as a non-invasive marker test for liver fibrosis, and found no relationship.

The lack of a relationship of markers of hepatic steatosis, inflammation and fibrosis with CV events in my study may be due to a number of reasons. Firstly, it may be that there is truly no relationship in the population under study. This cohort differs from many of those studied previously, mainly in its broad spectrum of patients with type 2 diabetes. Targher et al used populations for NAFL/D study derived exclusively from secondary care diabetes settings (spectrum bias), where the influence of hepatic steatosis would be expected to be stronger in more severe diabetes, consistent with other studies looking at more general populations and CV mortality[184]. Secondly, it may relate to specific cohort effects within my study, although the size and follow-up time are comparable with several other similar studies[189,200]. The association between the presence of steatosis and the absence of prevalent CVD may be explained, at least in part, by regression of hepatic steatosis

with advancing liver disease[388] or it may reflect survival bias, in that those with the most severe NAFL/D had already died prior to enrolment into the ET2DS. I note that my analysis takes into account all patients, not only those with USS diagnosed NAFL/D, however, other aetiologies of liver disease were excluded and few people drank alcohol to excess. Thirdly, it may reflect issues with the markers of liver injury under study in that they are not the most appropriate measures of advanced liver disease in a community cohort. Fourthly, there may truly be no relationship.

Given that no markers of liver injury were independently associated with CVD, this strengthens the argument for the GGT association being driven by systemic inflammation through the shared ectopic fat hypothesis, as opposed to a direct consequence of chronic liver disease (the atherogenic liver hypothesis).

The utility of different liver injury markers may be determined by the precise question being asked. For example, there is a body of evidence validating non-invasive liver markers for the cross-sectional stratification of liver disease in secondary care and predicting future liver-related clinical outcomes[389,390]. In this study, these markers do not appear to add prognostic benefit in determining cardiovascular end-points. However, GGT which is generally not considered useful for stratifying active liver disease does appear to be beneficial in determining prognostic CVD. Thus, one marker test or panel may not provide a diagnostic or prognostic panacea.

8.7 Future work

Given the wide variety of liver fibrosis markers identified (Chapter 2) and investigated (Chapters 5 and 6) is would have been most useful to determine the most accurate marker for identifying advanced fibrosis/cirrhosis in a community-based cohort. Without existing biopsy validation studies in a comparable cohort, or liver biopsy in this study, it is not possible to do this.

It has become increasingly evident during the time period that this work has been undertaken over, that whilst many people with type 2 diabetes will have hepatic

steatosis, predominantly due to NAFL/D and/or ALD, for the majority it will be a benign condition. The clinical questions that need to be answered are: i) who has clinically significant CLD and ii) who is going to get clinically significant CLD. This thesis contributes to answering these questions but there are several obstacles to answering them fully:

Using non-invasive markers to diagnose and/or predict the stages of non-alcoholic fatty liver disease or chronic liver disease

Currently this cannot be done. Markers are validated against an imperfect gold standard in select populations with poor PPVs. Moving forward, the most value is likely to come from the use of imaging modalities allowing the whole liver to be assessed for a number of different purposes.

Redefining the stages of 'advanced' chronic liver disease

The current histological grading systems are limited in their usefulness not only by the need for liver biopsy but also in their clinical utility. The move for example from Metavir F2 to the more severe Metavir F3, is not equal in magnitude to the move from Metavir F3 to F4, nor does a move in stage indicate useful clinical interpretation for developing hepatic failure and complications.

A more useful system would be to be able to provide a composite tool, most likely incorporating imaging alongside alternative markers allowing the categorisation of patients into those with: i) early disease at low risk of progression, ii) early disease at high risk of progression, iii) advanced disease amenable to treatment, iv) advanced disease not amenable to treatment, and v) end-stage disease (cirrhosis).

Defining chronic liver disease and robust clinical end-points in order to allow fair comparisons

Definitive diagnoses are needed to allow fair comparisons. What ICD-10 codes should be included in CLD? – as these current differ in many studies.

A more robust NAFLD diagnosis would be beneficial including formalising the components requiring exclusion for the diagnosis. For example, for hepatotoxic

medication use, what drugs, at what dose, over what time period? Also, what cut-off (and how should we measure) of alcohol consumption is clinically relevant i.e. does it matter if someone has ALD, NAFL/D or more commonly mixed ALD/NAFL/D?

Finally, what is the best way of defining progression to clinically significant chronic liver disease? For large scale population based studies, neither liver biopsy or reliance on hospital discharge coding is feasible or accurate.

Models for predicting CVD in people with type 2 diabetes

There are already both general population (Framingham Heart Study[391]) and diabetes specific (UK Pospective Diabetes Study[392]) risk prediction tools available for the prediction of CVD. However, the findings of this thesis suggest it may be useful to consider the addition of GGT into ascoring system for people with type 2 diabetes. Such a score would need to be tested in a large cohort with sufficient outcomes to see if the addition of GGT was beneficial, prior to further validation.

8.8 Conclusion

In conclusion, this thesis provides the first ever information on the 'normal' distribution of non-invasive markers of CLD in a diabetic population unselected for liver disease. Also, I have provided evidence that CK18 and ELF are increased in those people with type 2 diabetes who have a more adverse metabolic profile, including higher levels of body fat, whilst established risk factors for CLD were not found to have a major influence of levels of the markers. At present, choice of and use of these non-invasive markers is limited within low disease prevalence settings to excluding significant liver fibrosis. The practical and ethical challenges of large scale liver biopsy in 'normal' patients persist, but emerging techniques, such as magnetic resonance elastography[251], have the potential to become a more acceptable reference standard.

This investigation represents the only large prospective population-based study of clinically significant CLD in people with type 2 diabetes. Here for the first time, I have evaluated the utility of extensive investigation using routinely available clinical

tests in order to identify those at high risk of both immediate and future clinically significant CLD and finding a large burden of undiagnosed clinically significant CLD.

Ultimately these findings could help identify particularly high risk groups within the diabetic population who may benefit from increased surveillance in relation to development of CLD and/or from targeting of specific metabolic risk factors.

Prospective studies are now required to determine the extent to which non-invasive markers predict the development of clinically relevant liver-related endpoints, such as hepatocellular carcinoma, oesophageal varices and cardiovascular outcomes, in a range of different low and high risk population groups, most notably community settings and to identify additional risk factors responsible for the development of advanced liver disease in people with type 2 diabetes.

For maximal clinical impact future work needs to include continued longer term follow-up of the ET2DS cohort to capture future incident liver related events. Of particular interest will be how the rate of change of potential markers in earlier stages of CLD can be used to predict future development of clinically significant CLD.

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Appendices

Appendix A Classification of diabetes

Classification of diabetes mellitus according to the American Diabetes Association. Diabetes Care January 2014 vol. 37 no. Supplement 1 S81-S90.

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)
   A. Immune mediated
   B. Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)

III. Other specific types
   A. Genetic defects of β-cell function
      1. MODY 3 (Chromosome 12, HNF-1α)
      2. MODY 1 (Chromosome 20, HNF-4α)
      3. MODY 2 (Chromosome 7, glucokinase)
      4. Other very rare forms of MODY (e.g., MODY 4: Chromosome 13, insulin promoter factor-1; MODY 6: Chromosome 2, *NeuroD1;* MODY 7: Chromosome 9, carboxyl ester lipase)
      5. Transient neonatal diabetes (most commonly ZAC/HYAMI imprinting defect on 6q24)
      6. Permanent neonatal diabetes (most commonly KCNJ11 gene encoding Kir6.2 subunit of β-cell K_{ATP} channel)
      7. Mitochondrial DNA
      8. Others
   B. Genetic defects in insulin action
      1. Type A insulin resistance
      2. Leprechaunism
      3. Rabson-Mendenhall syndrome
      4. Lipoatrophic diabetes
      5. Others
   C. Diseases of the exocrine pancreas
      1. Pancreatitis
      2. Trauma/pancreatectomy
      3. Neoplasia
      4. Cystic fibrosis
      5. Hemochromatosis
      6. Fibrocalculous pancreatopathy
      7. Others
   D. Endocrinopathies
      1. Acromegaly
      2. Cushing's syndrome
      3. Glucagonoma
      4. Pheochromocytoma
      5. Hyperthyroidism
      6. Somatostatinoma
      7. Aldosteronoma
      8. Others
   E. Drug or chemical induced
      1. Vacor
      2. Pentamidine
      3. Nicotinic acid
      4. Glucocorticoids
      5. Thyroid hormone
      6. Diazoxide
      7. β-Adrenergic agonists
      8. Thiazides
      9. Dilantin
      10. γ-Interferon
      11. Others
   F. Infections
      1. Congenital rubella
      2. Cytomegalovirus
      3. Others
   G. Uncommon forms of immune-mediated diabetes
      1. Stiff-man syndrome
      2. Anti-insulin receptor antibodies
      3. Others
   H. Other genetic syndromes sometimes associated with diabetes
      1. Down syndrome
      2. Klinefelter syndrome
      3. Turner syndrome
      4. Wolfram syndrome
      5. Friedreich ataxia
      6. Huntington chorea
      7. Laurence-Moon-Biedl syndrome
      8. Myotonic dystrophy
      9. Porphyria
      10. Prader-Willi syndrome
      11. Others

IV. Gestational diabetes mellitus

Patients with any form of diabetes may require insulin treatment at some stage of their disease. Such use of insulin does not, of itself, classify the patient.

Appendix B Histological fibrosis staging systems

Table B-1 Histological staging systems for liver fibrosis

	Stage	*Fibrosis*
Scheuer	0	None
	1	Enlarged, fibrotic portal tracts
	2	Periportal or portal-portal septa but intact architecture
	3	Fibrosis with architectural distortion but no obvious cirrhosis
	4	Probable or definite cirrhosis
Ishak	0	No fibrosis
	1	Fibrous expansion of some portal areas, with or without short fibrous septa
	2	Fibrous expansion of most portal areas, with or without short fibrous septa
	3	Fibrous expansion of most portal areas with occasional P-P bridging
	4	Fibrous expansion of portal areas with marked bridging (P-P and P-C)
	5	Marked bridging (P-P and/or P-C) with occasional nodules
	6	Cirrhosis, probable or definite
Metavir	0	No fibrosis
	1	Stellate enlargement of portal tract but without septa formation
	2	Enlargement of portal tract with rare septa formation
	3	Numerous septa without cirrhosis
	4	Cirrhosis
Brunt	0	None
	1	Zone 3 perisinusoidal/pericellular fibrosis; focally or extensively present.
	2	Zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis
	3	Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis
	4	Cirrhosis

P-C portal-central; **P-P** portal to portal

Scheuer, P. J. (1991). "Classification of chronic viral hepatitis: a need for reassessment." Journal of Hepatology **13**(3): 372-374.

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Appendix C Systematic review – protocol

Background

Non-alcoholic fatty liver disease (NAFLD) is an increasingly recognised chronic liver disease characterised by fat deposition within the liver parenchyma. It requires the absence of significant alcohol consumption at levels thought to be harmful to the liver. It involves a wide spectrum of disease with histological findings ranging from fat deposition only, through inflammatory changes to fibrosis and cirrhosis[204]. Clinical difficulties arise as most patients with NAFLD have no clinical signs or symptoms until the disease has progressed to significant fibrosis, with a proportion progressing to end-stage liver disease and there is also an association with the development of hepatocellular carcinoma[205,206].

The true prevalence of NAFLD is unknown, however it is estimated to be between 6 and 14% in the general population[393], rising to up to 74% in obese individuals[394] . There are strong associations between NAFLD and the metabolic syndrome and experts in the field continue to argue the exact relationship with divided opinion as to whether NAFLD is a disease in its own right or a component of the metabolic syndrome[395].

Confirmation of hepatic fibrosis in NAFLD is currently only possible using liver biopsy [204]. This is an invasive investigation requiring a 6 hour inpatient observation period post procedure. Common minor complications include pain, nausea, and vomiting. Potential significant complications include haemorrhage, billiary leakage and transient jaundice, with a small but acknowledged mortality rate of <0.5%[150,207]. It is therefore not feasible to use liver biopsy recurrently for follow-up investigation of patients given its unfavourable nature.

Methods

Search strategy

The search objective is to identify all published original research studies examining the use of non-invasive methods for the diagnosis of hepatic fibrosis in patients with non-alcoholic fatty liver disease.

Search sources:

- An electronic database search will be conducted from inception to present date; MEDLINE, EMBASE. Global Health, Web of Science, SCIRUS and Cochrane.
- Additional studies will be identified via manual review of the reference lists of identified studies, review articles and citation searching using Web of Science Citation Index.
- From relevant websites: American Association for the Study of the Liver, European Association for the Study of the Liver.

Search terms employed are detailed below. MESH terms were used where available.

Criteria for inclusion

Non-invasive methods of diagnosing hepatic fibrosis

- Individual serum markers; or
- Marker panels must include ≥2 components; or
- Any imaging modality

Study characteristics

- Data on NAFLD extractable
- They are systematic reviews, meta-analyses, or primary studies of one or more non-invasive markers
- Written in English
- Liver biopsy as the reference standard
- >30 participants

NAFLD defined as a biopsy diagnosis with a statement regarding the attempt to exclude other causes of liver disease (e.g. alcoholic liver disease, viral hepatitis, autoimmune disorders, metabolic disorders). Studies where patients consumed alcohol in excess of 40g/day for men and 20g/day for women will be excluded.

Primary outcome of grade hepatic fibrosis as defined using any recognised histological classification.

Those studies with data reported as sensitivity, specificity, predictive values, likelihood ratios (LR), or ROC curves or included sufficient data to calculate these parameters will be included.

Data presented as to allow the construction of diagnostic 2x2 tables with the 4 cells; true positives, false negatives, false positives and true negatives for a defined diagnostic threshold.

Data collection

Data extraction will be undertaken by one reviewer (JM) and checked by a second reviewer (JP) with any disagreements being resolved through discussion.

Methodological quality assessment of the identified studies will be undertaken using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) score[255,256,396].

1. *Hyperlipidemia/ or *Hypertriglyceridemia/ or *Metabolic Syndrome X/ or *Obesity/ or *Insulin Resistance/ or *Diabetes Mellitus, Type 2/ or metabolic syndrome.mp.
2. fatty liver.mp. or exp Fatty Liver/
3. NAFLD.mp.
4. NASH.mp.
5. steatohepatitis.mp.
6. steatosis.mp.
7. non-alcoholic.mp.
8. 1 or 2 or 3 or 4 or 5 or 6 or 7
9. fibrosis.mp.
10. cirrhosis.mp.
11. $hepatitis.mp.
12. steatosis.mp. [mp=title, original title, abstract, name of substance word, subject heading word]
13. inflammation.mp.
14. 9 or 10 or 11 or 12 or 13
15. serum markers.mp. or exp Biological Markers/

16. diagnosis/ or "diagnostic techniques and procedures"/ or "laboratory techniques and procedures"/
17. $invasive.mp.
18. exp Liver Function Tests/
19. exp DIAGNOSTIC IMAGING/
20. ELASTOGRAPHY.MP
21. predict$.mp.
22. marker$.mp.
23. surrogate.mp.
24. 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 OR 23
25. 8 and 14 and 24
26. limit 25 to (humans and english language)
27. limit 26 to "all adult (19 plus years)"

Appendix D Systematic review data extraction forms

Study Selection, Quality Assessment & Data Extraction Form

First author	Journal/Conference Proceedings etc	Year

Study eligibility

Study of diagnostic test accuracy	Relevant participants (n>30, age≥18)	Liver biopsy gold standard	Appropriate outcomes reported
Yes / No / Unclear	Yes / No / Unclear	Yes / No / Unclear	Yes / No / Unclear

Do not proceed if any of the above answers are 'No'. Record reason for exclusion

References to study

Check other references identified in searches. If there are further references to this trial link the papers now & list below. All references to a single study should be linked.

Code	Author(s)	Journal/Conference Proceedings etc	Year
A	*The paper listed above*		
B	*Further papers*		

Participants and trial characteristics

Participant characteristics	
Age (mean, median, range, etc)	
Sex of participants (numbers / %, etc)	

Trial characteristics	
Single centre / multicentre	
Country / Countries	
Participant source (hepatology outpatient clinic etc)	

NAFLD exclusions (alcohol, hep B/C etc)	
How many people were included?	
Number of participants in each fibrosis group	
Number of participants who were analysed	
Histology system used (e.g. Brunt)	
Fibrosis cut-offs reported	
Single serum marker (name and manufacturer)	
Marker panel (name and manufacturer, algorithm)	
Imaging (type and manufacturer)	

Data extraction

Outcomes	
F0 vs F1-4	Yes / No
F0-1 vs F2-4	Yes / No
F0-2 vs F3-4	Yes / No
F0-3 vs F4	Yes / No

Diagnostic test accuracy								
Code	Fibrosis level	Cut-off	Sensitivity	Specificity	PPV	NPV	AUROC	Notes

Other information which you feel is relevant to the results

References to other studies

Did this report include any references to published reports **of potentially eligible studies not already identified for this review?**		
First author	Journal / Conference	Year of publication
Did this report include any references to **unpublished data** from potentially eligible studies not already identified for this review? If yes, give list contact name and details		

Appendix E PRISMA Checklist for systematic literature review: non-invasive markers of liver fibrosis in NAFL/D

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	✓
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	✓
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	✓
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	✓
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	✓
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	✓
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	✓
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	✓
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	✓
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	✓

Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	✓
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	✓
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	N/A
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	✓
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	✓
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	✓
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	✓
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	✓
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	✓
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	✓
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	✓
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	✓
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	✓
FUNDING			

Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

Appendix F Systematic review of non-invasive markers of liver fibrosis: additional tables

Table F-1 Excluded studies n=16

Study	*Location*	*Date*	*Reason for exclusion*
Blomme 2010[257257257253254247]	Belgium	2010*	No AUROC presented
Casey 2010[25825825825425524823 4](Casey et al.)(Casey et al.)(234)[234][234][234][234234234233231221]	Australia	2010*	Biopsy only performed in a subgroup
Chen 2010	USA	2007-2010	No AUROC presented
De Ledinghen 2010	France	2008	No liver biopsy
Fujii 2009	Japan	1998-2007	Only report AUROC for cirrhosis (F4)
Hartleb 2005	Poland	1995-2002	No AUROC presented
Kang[$] 2007	USA	2007*	No AUROC presented
Khosravi 2011	Iran	2005-2009	No AUROC presented
Kolesnikova 2008	Ukraine	2008*	No AUROC presented
Laine 2004	France	2000-2003	Unable to separate NAFLD and ALD
Pimentel 2010	Brazil	2010*	No AUROC presented
Qureshi 2008	USA	2002-07	No AUROC presented
Schmilovitz-Weiss 2008	Israel	2008*	N<30
Tobari 2009	Japan	1990-2007	No AUROC presented
Wong 2009	Hong Kong	2009	No AUROC presented
Yoneda 2010[270270270266267260]	Japan	2008	Repeat publication of data

Table E-2 Publication years of included studies

2000	*2001*	*2002*	*2003*	*2004*	*2005*	*2006*	*2007*	*2008*	*2009*	*2010*	*2011*	*2012**
1	**0**	**0**	**0**	**3**	**6**	**6**	**6**	**9**	**9**	**6**	**5**	**1**

***only for the period Jan-March.**

Appendix G Systematic review of non-invasive markers of liver fibrosis: quality assessment of included studies

Table G-1 Quality scoring of included studies: QUADAS-2

Study	*Risk of bias*					
	Patient selection	Index test	Reference standard	Flow and timing	Overall	Reason for risk of bias
Angulo[243]	✓	✓	✓	✓	None	
Cales[272]	✓	✓	✓	✓	None	
Guha[236]	✓	✓	✓	✓	None	
Park[296]	✓	✓	✓	✓	None	
Petta[297]	✓	✓	✓	✓	None	
Ruffillo[301]	✓	✓	✓	✓	None	
Suzuki[305]	✓	✓	✓	✓	None	
Wong[246]	✓	✓	✓	✓	None	
Freidrich-Rust[275]	✓	✓	✓	×	Risk	Interval between index and reference test 18 months
Gaia[276]	✓	✓	✓	×	Risk	Interval between index and reference test 6 months
Gholam[277]	×	✓	✓	✓	Risk	All gastric bypass surgery participants
Guajardo-Salinas[278]	×	✓	✓	?	Risk	All gastric bypass surgery participants Required insurance clearance for index test
Kallwitz[279]	×	✓	✓	?	Risk	All gastric bypass surgery participants
Kelleher[282 $]	×	✓	✓	?	Risk	All NASH participants
Lassailly[284]	×	✓	✓	?	Risk	All gastric bypass surgery participants
Lupsor[285]	×	✓	✓	✓	Risk	All NASH participants
Lydatakis[286]	×	✓	✓	?	Risk	All NASH participants
McPherson[228]	✓	✓	✓	×	Risk	Interval between index and reference test 6 months
Miao[288$]	×	✓	✓	?	Risk	All participants morbidly obese
Myers[291]	?	✓	✓	×	Risk	Interval between index and reference test 6 months
Myers[292]	×	✓	✓	×	Risk	All participants morbidly obese Interval between index and reference test 6 months
Tetri[293]	×	✓	✓	×	Risk	Some participants selected from

Study	*Risk of bias*					
						PIVENs clinical trial – multiple exclusion criteria. Interval between index and reference test 6 months
Ratziu[119]	×	✓	✓	✓	Risk	All participants obese
Ratziu[241]	×	✓	✓	?	Risk	Hospitalised patients only
Rodriguez[299]	×	✓	✓	✓	Risk	All gastric bypass surgery participants
Rosenberg[235]	✓	✓	✓	×	Risk	Interval between index and reference test 6 months
Shah[303]	?	✓	✓	×	Risk	Interval between index and reference test 12 months
Talwalker[306$]	?	?	?	×	Risk	Interval between index and reference test 12 months
Wong[309]	×	✓	✓	✓	Risk	Chinese population
Yoneda[311]	×	✓	✓	?	Risk	All NASH participants
Yu[314]	×	✓	✓	?	Risk	Included a group of liver donors
Adams[271$]	?	✓	✓	✓	Unclear	
de Ledinghen[273]	✓	✓	✓	?	Unclear	
Dos Santos[274]	?	✓	✓	?	Unclear	
Fujii[234 #]	?	✓	✓	?	Unclear	
Harrison[233]	?	✓	✓	?	Unclear	
Kaneda[280]	✓	✓	✓	?	Unclear	
Kayadibi[281]	?	✓	✓	?	Unclear	
Kruger[283]	?	✓	✓	?	Unclear	
Loaeza[229]	?	✓	✓	?	Unclear	
Malik[86]	✓	✓	✓	?	Unclear	
Manousou[287]	?	✓	✓	✓	Unclear	
Miette[289]	?	?	?	?	Unclear	
Miyaaki[290]	✓	✓	✓	?	Unclear	
Palekar[294]	?	✓	✓	?	Unclear	
Palmeri[295]	✓	✓	✓	?	Unclear	
Ratziu[298$]	?	?	?	?	Unclear	
Rosenberg[300$]	✓	✓	✓	?	Unclear	
Sakugawa[302]	✓	✓	✓	?	Unclear	
Sumida[304]	✓	✓	✓	?	Unclear	
Tropet[307$]	?	?	?	?	Unclear	
Wong[308$]	✓	?	✓	✓	Unclear	
Yilmaz[310]	?	✓	✓	?	Unclear	

Study	*Risk of bias*				
Yoneda[247]	✓	✓	✓	?	Unclear
Yoneda[247]	?	✓	✓	✓	Unclear
Yoneda[312#]	?	✓	✓	?	Unclear
Younossi[313]	✓	✓	✓	?	Unclear

Appendix H Study questionnaires

EDINBURGH TYPE 2 DIABETES STUDY

BASELINE

QUESTIONNAIRE

PLEASE NOTE: ONE OF OUR RESEARCH NURSES WILL GO OVER THE QUESTIONNAIRE WITH YOU AT THE CLINIC AND MAY ASK A FEW ADDITIIONAL QUESTIONS

THE INFORMATION IN THIS QUESTIONNAIRE IS HIGHLY CONFIDENTIAL AND IS PART OF A MEDICAL RESEARCH STUDY

The information you give in this questionnaire will be treated as strictly confidential and will be available only to your own doctor and the study team. The results of the research will appear only in the form of general statistics from which it will be impossible to identify you as an individual.

Please complete the following:

SURNAME: ……..

FORENAMES: ……..

DATE: ……..

If you have any difficulties in answering some of the questions, you will have a chance to discuss these with a member of the study team.

THANK YOU FOR YOUR CO-OPERATION IN THIS STUDY

For Office Use: Study No….....

BASELINE QUESTIONNAIRE

IT IS IMPORTANT TO ANSWER ALL THE QUESTIONS CAREFULLY. PLEASE TAKE YOUR TIME.

PERSONAL HISTORY

1. Please tick one box: Male ☐ Female ☐

2. Enter your date of birth: Day ☐☐ Month ☐☐ Year ☐☐

3. Please tick the box showing your present marital status:

- Married and/or living with long-term partner ☐
- Single ☐
- Widowed ☐
- Divorced or separated ☐

4. Please enter your address (including postcode) and telephone no.

Address: …….

…….

Postcode: ……..

Telephone no: ……

5. Please enter the details of your GP

GP name: …….

Address: …….

…….

EDUCATION

6. What is the HIGHEST level of education you and your spouse/ex-spouse or long-term partner have completed?

Please tick appropriate boxes:

	You	Spouse/ex-/partner
University/college degree course	☐	☐
Other professional/technical qualification after leaving school	☐	☐
Secondary school	☐	☐
Primary school	☐	☐

ETHNICITY

7. What is your ethnic group?

Please choose ONE section from 1 to 5, then tick the appropriate box to indicate your ethnic Group

(i) White

☐ British
☐ Any Other White background, *please write in* _____

(ii) Mixed

☐ White and Black Caribbean
☐ White and Black African
☐ White and Asian
☐ Any Other Mixed background, *please write in* _____

(iii) Asian or Asian British

☐ Indian
☐ Pakistani
☐ Bangladeshi
☐ Any Other Asian background, *please write in* _____

(iv) Black or Black British

☐ Caribbean
☐ African
☐ Any Other Black background, *please write in* _____

(v) Chinese or other ethnic group

☐ Chinese
☐ Any Other, *please write in* _____

CURRENT EMPOYMENT STATUS

8. At the moment, what is the employment status of you and your spouse/ex-spouse or long-term partner?

You		**Spouse/ex-spouse/partner**	
Employed, full-time	☐	Employed, full-time	☐
Employed, part-time	☐	Employed, part-time	☐
Unemployed	☐	Unemployed	☐
Retired	☐	Retired	☐
A Housewife (full-time)	☐	A Housewife (full-time)	☐
Other	☐	Other	☐
please specify ……..		please specify ..……	

MEDICAL HISTORY

Diabetes history

9. When was your diabetes diagnosed (if known)? Year ……..……

10. What treatment do you receive currently for your diabetes?

(i) Tablets Yes ☐ No ☐

If 'yes', please give name(s) …….....

(ii) Insulin injections Yes ☐ No ☐

If 'yes',

(a) give total number of units per day ……units/day

(b) give date (year) when you started insulin year ……

11. Have you **ever** had an episode of low blood glucose (hypoglycaemia) when you have needed **someone else** to treat you eg. give sugary drink or glucagon? Yes ☐ No ☐ Don't know ☐

If 'yes', how many times has this ever happened?

1-2 ☐
3-4 ☐
5 or over ☐

How many times has this happened **over the past year**?

1-2 ☐
3-4 ☐
5 or over ☐

12. Are you on any regular medical treatment from a doctor as follows:

	Yes	No	Don't Know
Aspirin?	☐	☐	☐
Drugs for angina (including spray)?	☐	☐	☐
Drugs to lower blood pressure?	☐	☐	☐
Drugs to lower cholesterol?	☐	☐	☐

(If you have answered YES to any of these, please include details below)

13. Give names of <u>all</u> current medication if possible (including regular skin creams, eye drops, inhalers, tablets and injections which may or may not be repeat prescriptions):

…… ……....
…… ……....
…… ……....

…… ……...

14. Have you taken any oral steroids, used steroid inhalers or used steroid containing creams or eye drops in the last 3 months?

Yes	No	Don't Know
☐	☐	☐

Vascular Disease

15. Have you ever been <u>told by a doctor</u> that you have or have had any of the following?

	Yes	No	Don't Know
(i) Heart attack (coronary thrombosis, myocardial infarction)?	☐	☐	☐
(ii) Angina?	☐	☐	☐
(iii) Stroke?	☐	☐	☐
(iv) Hardening of the arteries in the legs?	☐	☐	☐
(v) High blood pressure?	☐	☐	☐

If you have answered 'yes' to any of the above, please give the year in which the event occurred and/or condition was diagnosed (as near as you can remember) and the name of the hospital/GP surgery where you were/are treated for the condition

Event/condition	Year of event/diagnosis	Hospital/GP surgery where treated
……	…….	…….
……	……..	…….

16. Have you ever undergone any of the following procedures/operations?

	Yes	No	Don't Know
(i) An operation or balloon treatment to relieve a blockage in the arteries of your <u>heart</u> (coronary by-pass or angioplasty)?	☐	☐	☐
(ii) An operation or balloon treatment to relieve a blockage in the arteries of your <u>leg(s)</u> , other than for varicose veins?	☐	☐	☐
(iii) Surgery to remove toes or leg (above or below the knee)?	☐	☐☐	
(iv) An operation or balloon treatment to relieve a blockage in the arteries of your neck (carotid surgery/angioplasty/stenting)?	☐	☐	☐

If you have answered 'yes' to any of the above, please give the year in which the procedure was performed and the name of the hospital you attended

Procedure/operation	Year performed	Hospital attended
……	…….	…….
……	……..	…….

Liver Condition/Disease

17. Have you ever been <u>told by a doctor</u> that you have or have had any of the following?

		Yes	No	Don't Know
(i)	Hepatitis?	☐	☐	☐
(ii)	Cirrhosis of the liver?	☐	☐	☐
(iii)	Any other disease/medical condition affecting the liver?	☐	☐	☐

If you have answered 'yes' to any of the above, please give the name of the condition, the year in which it was diagnosed (as near as you can remember) and the name of the hospital where you were/are treated for the condition

Name of condition	Year of diagnosis	Hospital where treated
……	…….	…….
……	……..	…….

18. Have you ever had any of the following investigations of your liver

		Yes	No	Don't Know
(i)	Abnormal blood tests of liver function?	☐	☐	☐
(ii)	Liver biopsy?	☐	☐	☐
(iii)	Scan (ultrasound or CT etc.) of the liver?	☐	☐	☐
(iv)	Other investigation of the liver?	☐	☐	☐

If you have answered 'yes' to any of the above, please give the name of the investigation, the year in which it was done (as near as you can remember) and the name of the hospital where the test/investigation was performed

Name of investigation	Year done	Hospital where performed
……	…….	…….
……	……..	…….

Other Medical Conditions

		Yes	No	Don't Know
19.	Do you suffer from disease of the thyroid gland?	☐	☐	☐
20.	Do you have any other medical conditions not mentioned above?	☐	☐	

If yes, please specify: ……..

……

……..

ALCOHOL

21. **Current alcohol intake**

(i) Think back carefully over the last seven days. Please write in each column the exact number of alcoholic drinks you consumed on each day during the past week. If none consumed write '0' in the boxes.

Try to remember where and who you were with on each day. This may help you remember what you had to drink.

	Pints of beer, lager, cider etc	Single glasses of whisky, vodka, gin etc	Single glasses of martini, wine, sherry, etc
Monday	☐	☐	☐
Tuesday	☐	☐	☐
Wednesday	☐	☐	☐
Thursday	☐	☐	☐
Friday	☐	☐	☐
Saturday	☐	☐	☐
Sunday	☐	☐	☐

(ii) Would you say that last week was fairly typical of what you usually have to drink in a week? Yes ☐ No ☐

(iii) If last week was not typical, would you normally drink more or less in a week? More ☐ Less ☐

22. **Alcohol intake over past year**

(i) 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).

- never ☐
- monthly or less ☐
- 2 to 4 times a month ☐
- 2 to 3 times a week ☐
- 4 to 5 times a week ☐
- 6 or more times a week ☐

(ii) How many drinks did you have on a typical day when you were drinking in the past year?

- 0 drinks ☐
- 1 to 2 drinks ☐
- 3 to 4 drinks ☐
- 5 to 6 drinks ☐
- 7 to 9 drinks ☐

10 or more drinks ☐

(iii) How often did you have 6 or more drinks on one occasion in the past year?

never ☐
less than monthly ☐
monthly ☐
weekly ☐
daily or almost daily ☐

23. Have you or your doctor ever considered that you suffer/have suffered in the past from an alcohol problem/excessive drinking? Yes ☐ No ☐

SMOKING

Smoking has been linked with many health problems. It is important that you answer the following section as accurately as possible.

24. Do you smoke at present? Yes ☐ No ☐

If no, proceed to Question 29

25. What do you usually smoke now?

	Yes	No
Cigarettes	☐	☐
Pipe	☐	☐
Cigars	☐	☐

26. How many do you usually smoke now?

Cigarettes per day …… cigarettes

Ozs. tobacco per week …… ozs.

Cigars per week …… cigars

27. For how many years during your life have you smoked cigarettes? …… years

28. How many cigarettes have you smoked on average per day during the period you have smoked? ……cigarettes

Now proceed to Question 34

29. Have you ever smoked regularly? Yes ☐ No ☐

If no, proceed to Question 34

30. What did you usually smoke? Yes ☐ No ☐

Cigarettes

Pipe ☐ ☐

Cigars ☐ ☐

31. How much did you smoke on average while you were a smoker?

Cigarettes per day …… cigarettes

Ozs. tobacco per week …… oz.

Cigars per week …… cigars

32. For how many years did you smoke cigarettes? …… years

33. If you smoked cigarettes, how long is it since you finally gave up?

……. years ……. months

CHEST PAIN

34. Do you ever get pain or discomfort in your chest? Yes ☐ No ☐

IF NO, PROCEED TO QUESTION 40

35. Do you get this pain or discomfort when you walk uphill or hurry? Yes ☐ No ☐

IF NO, PROCEED TO QUESTION 40

36. Do you get it when you walk at an ordinary pace on the level? Yes ☐ No ☐

37. When you get any pain or discomfort in your chest what do you do?

Tick one

Stop ☐

Slow down ☐

Continue at the same pace ☐

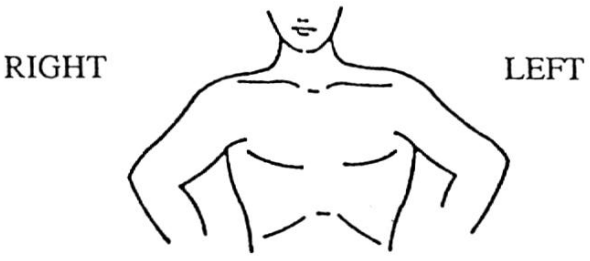
38. Does it go away when you stand still or sit down? Yes ☐ No ☐

How soon? Tick one

10 minutes or less ☐

More than 10 minutes ☐

39. Where do you get this pain or discomfort? Mark the place(s) with an 'X' on the diagram



40. (i) Have you ever had a severe pain across the front of your chest lasting for half an hour? Yes ☐ No ☐

(ii) What was the cause? ……

LEG PAIN

		Yes	No	I am unable to walk
41.	Do you get a pain or discomfort in your leg(s) when you walk?	☐	☐	☐

If you answered 'yes' to question 41, please answer the following questions.

	Yes	No
(i) Does this pain ever begin when you are standing still or sitting?	☐	☐
(ii) Do you get it if you walk uphill or hurry?	☐	☐
(iii) Do you get it when you walk at an ordinary pace on the level?	☐	☐
(iv) Does the pain ever disappear while you are still walking?	☐	☐
(v) What do you do if you get it when you are walking?	☐	☐

	Tick one
Stop	☐
Slow down	☐
Continue at same pace	☐

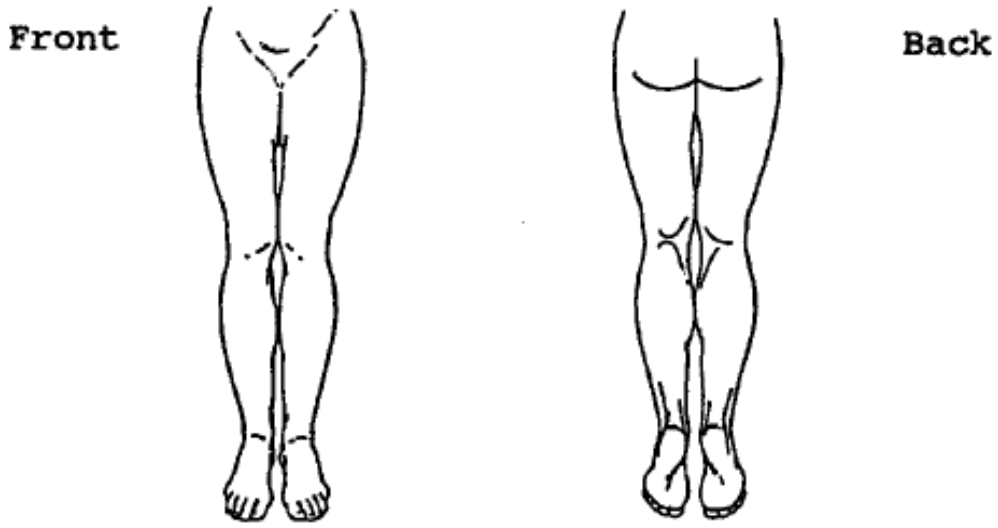
(vi) What happens to it if you stand still?

	Tick one
Usually continues for more than 10 minutes	☐
Usually disappears in 10 minutes or less	☐

(vii) Where do you get this pain or discomfort?

	Yes	No
(i) Do you get this pain in your calf (or calves)?	☐	☐

(ii) Please mark the place(s) where you get the pain with 'X' on the diagram below



THANK YOU FOR COMPLETING THIS QUESTIONNAIRE – PLEASE BRING IT WITH YOU TO YOUR APPOINTMENT AT THE WELLCOME TRUST CLINICAL RESEARCH FACILITY

EDINBURGH TYPE 2 DIABETES STUDY

YEAR 1 QUESTIONNAIRE

(LIVER FUNCTION & DIABETES COMPLICATIONS)

THE INFORMATION IN THIS QUESTIONNAIRE IS HIGHLY CONFIDENTIAL AND IS PART OF A MEDICAL RESEARCH STUDY.

The information you give in this questionnaire will be treated as strictly confidential and will be available only to your own doctor and the study team. The results of the research will appear only in the form of general statistics from which it will be impossible to identify you as an individual.

Please complete the following :

SURNAME : ……..

FORENAMES : ……..

DATE : ……..

If you have any difficulties in answering some of the questions, you will have a chance to discuss these with a member of the study team.

Thank you for your continued participation in the Edinburgh Type 2 Diabetes Study

PART 1

1. Medications

(a) What medications are you taking at present?

	Office Use Only
1……	☐
2……	☐
3……	☐
4……	☐
5……	☐
6……	☐
7……	☐
8……	☐
9……	☐
10……	☐

(b) Have you, to your knowledge, used any of the following medications over the last six months?

	Yes	No	Not sure
Amiodarone	☐	☐	☐
Isoniazid	☐	☐	☐
Methotrexate	☐	☐	☐
Steroids (e.g. prednisolone, dexamethasone)	☐	☐	☐
Allopurinol	☐	☐	☐
Tamoxifen	☐	☐	☐

If so, please indicate when it was started and how long you were on it.

Medication	**When started?**	**How long on it?**
_____	_____	_____
_____	_____	_____

2. Alcohol Intake

(a) Current alcohol intake

Think back carefully over the last seven days. Please write in each column exactly the number of alcoholic drinks you consumed on each day during the past week. If none consumed write '0' in the boxes.

Try to remember where and who you were with on each day. This may help you remember what you had to drink.

	Pints of beer, lager, cider etc	Single glasses of whisky, vodka, etc.	Single glasses of martini, wine, sherry, etc
Monday	☐	☐	☐
Tuesday	☐	☐	☐
Wednesday	☐	☐	☐
Thursday	☐	☐	☐
Friday	☐	☐	☐
Saturday	☐	☐	☐
Sunday	☐	☐	☐

(b) Alcohol intake over past year

(i) 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).

- never ☐
- monthly or less ☐
- 2 to 4 times a month ☐
- 2 to 3 times a week ☐
- 4 to 5 times a week ☐
- 6 or more times a week ☐

(ii) How many drinks did you have on a typical day when you were drinking in the past year?

- 0 drinks ☐
- 1 to 2 drinks ☐
- 3 to 4 drinks ☐
- 5 to 6 drinks ☐
- 7 to 9 drinks ☐
- 10 or more drinks ☐

(iii) How often did you have 6 or more drinks on one occasion in the past year?

- never ☐
- less than monthly ☐
- monthly ☐
- weekly ☐
- daily or almost daily ☐

(c) Have you or your doctor ever considered that you suffer/ have suffered in the past from an alcohol problem/excessive drinking?

Yes ☐ No ☐

3. Liver condition/disease

(a) In the past year (i.e. since we first saw you at the research clinic), have you been told by a doctor that you have any of the following?

		Yes	No	Not sure
(i)	Fatty liver?	☐	☐	☐
(ii)	Hepatitis?	☐	☐	☐
(iii)	Cirrhosis of the liver?	☐	☐	☐
(iv)	Any other medical condition affecting the liver?	☐	☐	☐

If you have answered 'yes' to any of the above, please give the name of the condition, when it was diagnosed and the name of the hospital where you were/are treated for the condition

Name of condition	When diagnosed	Hospital where treated
……	……	……
……	……	……..

(b) Have you had any of the following investigations of your liver within the last year?

		Yes	No	Not sure
(v)	Abnormal blood tests of liver function?	☐	☐	☐
(vi)	Liver biopsy?	☐	☐	☐
(vii)	Scan (ultrasound or CT etc.) of the liver?	☐	☐	☐
(viii)	Other investigation of the liver?	☐	☐	☐

If you have answered 'yes' to any of the above, please give the name of the investigation, when it was done and the name of the hospital where the test/investigation was performed

Name of investigation	When done	Hospital where performed
……	……	……
……	……	……..

4. Joint condition/disease?

Have you ever been told by a doctor that you have any of the following?

		Yes	No	Not sure
(i)	Osteoarthritis	☐	☐	☐
(ii)	Rheumatoid arthritis	☐	☐	☐
(iii)	Scleroderma	☐	☐	☐
(iv)	Any other disease affecting the joints	☐	☐	☐

If you have answered 'yes' to any of the above, please give the name of the condition, when it was diagnosed and the name of the hospital where you were/are treated for the condition

Name of condition	When diagnosed	Hospital where treated
……	……	……
……	……	……..

PART 2

Some people find that they have altered breathing when they are asleep, sometimes leading to snoring. It has been suggested that this may affect their thinking skills, such as those which we measured when you first attended our research clinic. We are interested in finding out more about this condition and would therefore be grateful if you would answer the following questions.

Do you snore?

☐ Yes
☐ No **(If NO, go to Question 5)**
☐ Don't know

If you snore:

Your snoring is:

☐ Slightly louder than breathing
☐ As loud as talking
☐ Louder than talking
☐ Very loud – can be heard in adjacent rooms

How often do you snore?

☐ Nearly every day
☐ 3-4 times per week
☐ 1-2 times per week
☐ 1-2 times per month
☐ Never or nearly never

Has your snoring ever bothered other people?

☐ Yes
☐ No
☐ Don't know

Has anyone noticed that you stop breathing during your sleep?

☐ Nearly every day
☐ 3-4 times per week
☐ 1-2 times per week
☐ 1-2 times per month
☐ **Never or nearly never**

How often do you feel tired or fatigued after your sleep?

☐ Nearly every day
☐ 3-4 times per week
☐ 1-2 times per week
☐ 1-2 times per month
☐ Never or nearly never

During your waking time, do you feel tired, fatigued or not up to par?

☐ Nearly every day
☐ 3-4 times per week
☐ 1-2 times per week
☐ 1-2 times per month
☐ Never or nearly never

Have you ever nodded off or fallen asleep while driving a vehicle?

☐ Yes
☐ No

How often does this occur?

☐ Nearly every day
☐ 3-4 times per week
☐ 1-2 times per week
☐ 1-2 times per month
☐ Never or nearly never

Do you have high blood pressure?

☐ Yes
☐ No
☐ Don't know

11. Sleepiness Score

How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired? This refers to your usual way of life in recent times. Even if you have not done some of these things recently try to work out how they would have affected you. Use the following scale to choose the most appropriate number for each situation.

0 = would *never* dose
1 = *slight* chance of dozing
2 = *moderate* chance of dozing
3 = *high* chance of dozing

SITUATION	CHANCE OF DOZING
Sitting and reading	
Watching television	
Sitting inactive in a public place (*e.g. a theatre or meeting*)	
As a passenger in the car for an hour without a break	
Lying down to rest in the afternoon when circumstances permit	
Sitting and talking to someone	
Sitting quietly after a lunch without alcohol	
In a car, while stopped for a few minutes in the traffic	

PART 3: DIABETES SERVICES

We would like to ask you some questions about the care that you receive for your diabetes. This part of the questionnaire has been developed by the research team and clinicians involved in the care of people with diabetes in Lothian. The aim is to provide feedback and information which will help improve the quality of care provided for people with diabetes. Please answer the questions as carefully as possible. Your responses will remain confidential and will not be disclosed to anyone involved in the care of your diabetes at an individual level.

A. Visits to the doctor or nurse

Q1. Where do you usually go for your diabetes check-up, where your test results and treatment are reviewed? This check-up is sometimes known as an 'annual review' though some people have more or less than one every year (please tick *one box only*)

☐ My doctor's surgery

☐ The hospital clinic (please write in name of hospital)

☐ Somewhere else (please write in)

☐ It varies

☐ I have recently changed where I go for my check ups (please specify)

☐ I have never had a diabetes check-up

☐ Don't know

Q2. How often do you usually have a diabetes check-up?

☐ Approximately once a year

☐ Approximately once every 6 months

☐ Approximately once every 3 months

☐ More than once every 3 months

☐ Less than once a year

☐ I have never had a check-up

☐ Don't know

Q3. Where do you go if you need help or advice from healthcare staff to manage your diabetes in between your scheduled check-ups (please tick all that apply)?

- ☐ My general practitioner
- ☐ The practice nurse
- ☐ The diabetes specialist nurse
- ☐ The hospital clinic
- ☐ NHS 24
- ☐ A community pharmacist
- ☐ I never contact healthcare staff between check-ups
- ☐ Other healthcare professional (Please write in)

Q4. Overall, who have you seen for diabetes care in the last year (please tick all that apply)?

- ☐ The hospital consultant
- ☐ A hospital registrar
- ☐ My general practitioner
- ☐ The practice nurse
- ☐ The diabetes specialist nurse
- ☐ A podiatrist
- ☐ A dietician
- ☐ Other healthcare professional (Please write in)

Q5. Who do you see most often about your diabetes (please tick one box only)?

- ☐ The hospital consultant
- ☐ A hospital registrar
- ☐ My general practitioner
- ☐ The practice nurse
- ☐ The diabetes specialist nurse
- ☐ Other healthcare professional (Please write in)

Q6. Who is your lead healthcare professional; the person that you perceive to be in overall charge of your diabetes care (please tick one box only)?

- ☐ The hospital consultant
- ☐ My general practitioner
- ☐ The practice nurse
- ☐ The diabetes specialist nurse
- ☐ Other healthcare professional (Please write in)

- ☐ I don't know which healthcare professional is in overall charge of my care

Q7. At your doctor's surgery, which of the doctors or nurses have a special interest in diabetes (please tick *all* that apply)?

- ☐ My general practitioner
- ☐ One of the other general practitioners
- ☐ One of the nurses
- ☐ None of the doctors or nurses
- ☐ Don't know

Q8. How do you feel about where you currently go for your diabetes check-ups, where your test results and treatment are reviewed (please tick the box which applies most to you)?

- ☐ I am happy having my check-ups where I go at the moment (⇒ **Go To Q10)**
- ☐ I would prefer to have my check-ups at a hospital clinic
- ☐ I would prefer to have my check-ups at my doctor's surgery
- ☐ I would prefer to have my check-ups somewhere else (please write in)

Q9. If you would prefer to have your diabetes check-ups somewhere other than where you go at present, why is this (please tick *all* that apply)?

- ☐ I would find my check-ups easier to get to
- ☐ I would be more likely to see the same doctor or nurse each time
- ☐ I would be able to get all my checks and tests done at the same time
- ☐ I would be able to see an expert in diabetes care
- ☐ Other (please write in)

B. Information and communication

Q10 Thinking about **the last 12 months**, when you received care for your diabetes….

(a) How would you describe the overall amount of information you received about your diabetes from your healthcare professional(s)?

- ☐ I didn't receive any information
- ☐ I received about the right amount of information
- ☐ I received too much information
- ☐ I didn't want any information

(b) How do you feel about the amount of written information that you received?

- ☐ I received about the right amount of written information
- ☐ I would like more written information
- ☐ I would like less written information
- ☐ I didn't want any written information

(c) Were you given personal advice about the kinds of food to eat?

- ☐ Rarely or not at all
- ☐ Some of the time
- ☐ Almost always

(d) Were you given personal advice about your levels of physical activity?

- ☐ Rarely or not at all
- ☐ Some of the time
- ☐ Almost always

(e) In the last 12 months, which of the following, if any, would you have liked to receive more information about (Please tick *all* that apply)?

- ☐ Diet
- ☐ Medications
- ☐ Physical activity
- ☐ Blood glucose monitoring
- ☐ Sources of support
- ☐ Footcare
- ☐ Illnesses associated with diabetes, such as eye disease
- ☐ What is likely to happen to me because of my diabetes in the future
- ☐ What kind of services I should receive for my diabetes
- ☐ Other (please write in)

- ☐ I don't want any more information

Q11. In addition to any information you receive from your doctor, nurse or other healthcare professional about your diabetes, where else do you get information and/or discuss your diabetes (Please tick *all* that apply)?

- ☐ Diabetes UK or other helpline
- ☐ Internet
- ☐ Magazine or newspaper articles
- ☐ Friends or work colleagues
- ☐ Relatives
- ☐ I don't receive any other information or discuss diabetes with anyone else
- ☐ Other (please write in)

Q12. Thinking about your diabetes **<u>check-up</u> or annual review** (where your test results and treatment are reviewed) ……

(a) Do you feel that the doctor/nurse you see for your diabetes <u>check-up</u> has the right type of expertise and experience to manage your diabetes?

- ☐ Yes or almost certainly yes
- ☐ Possibly
- ☐ No or probably not
- ☐ Don't know

(b) Do you usually see the same doctor/nurse each time for your diabetes <u>check up</u>?

- ☐ Always or almost always
- ☐ Sometimes
- ☐ Rarely or never
- ☐ Don't know

(c) How important is it to you that you should see the same doctor or nurse each time?

- ☐ Very important
- ☐ Quite important
- ☐ Not very important
- ☐ Don't know

(d) Is it easy and convenient for you to get to your diabetes <u>check-ups</u>?

- ☐ Always or almost always
- ☐ Sometimes
- ☐ Rarely or never
- ☐ Don't know

(e) Do you feel that you have sufficient time with the doctor or nurse when you see them for your diabetes check up?

- ☐ Always or almost always
- ☐ Sometimes
- ☐ Rarely or never
- ☐ Don't know

(f) Do you understand what is said at your diabetes check up?

- ☐ Always or almost always
- ☐ Sometimes
- ☐ Rarely or never
- ☐ Don't know

(g) Would you like to have a written copy of what is discussed and of any decisions that are made at your diabetes check-up?

- ☐ I would definitely like a written copy of all check-ups
- ☐ I could be interested in a written copy but do not feel strongly about it
- ☐ I do not want a written copy of my check-ups
- ☐ Don't know

(h) Do you feel that the doctor/nurse takes your concerns into account at your diabetes check-up?

- ☐ Always or almost always
- ☐ Sometimes
- ☐ Rarely or never
- ☐ Don't know

(i) Do you feel that the advice given at your diabetes check-up is tailored to your needs and to your ability to act on that advice?

- ☐ Always or almost always
- ☐ Sometimes
- ☐ Rarely or never
- ☐ Don't know

(j) Do you feel that you are involved in the decision making process around your diabetes care during your diabetes check up?

- ☐ Always or almost always
- ☐ Sometimes
- ☐ Rarely or never
- ☐ I don't want to be involved

(k) Do you have a management plan?

☐ Yes, verbal and written
☐ Yes, verbal only
☐ No
☐ Don't know

Q13. In general, do you feel that the health professionals looking after you have enough information about you eg. do they have your up-to-date records to refer to?

☐ Always or almost always
☐ Sometimes
☐ Rarely or never
☐ Don't know

C. Tests

Q14. It is likely that from time to time you will have a special blood test taken to look at your long-term or 'average' blood glucose level. This test is called HbA1c. Thinking about the last time you had this blood test:

(a) Do you know your HbA1c value?

☐ Yes
☐ No
☐ I don't want to know my HbA1c test value
☐ I have not had an HbA1c test (⇒ **Go To Q15)**

(b) Were you given your HbA1c test results in writing?

☐ Yes
☐ No
☐ I did not want the result in writing
☐ Don't know

(c) Do you have a target HbA1c?

☐ Yes
☐ No
☐ Don't know

Q15. Thinking about the last time you had your blood pressure measured:

(a) Do you know what your last blood pressure reading was?

☐ Yes
☐ No
☐ I don't want to know my blood pressure reading

☐ I have not had my blood pressure measured (⇒ **Go To Q16)**

Were you given your blood pressure results in writing?

☐ Yes
☐ No
☐ I did not want the result in writing
☐ Don't know

(c) Do you have a target blood pressure?

☐ Yes
☐ No
☐ Don't know

Q16. Thinking about the last time you had a blood test to measure your cholesterol level:

Do you know the result of your cholesterol test?

☐ Yes
☐ No
☐ I don't want to know the result
☐ I have not had my cholesterol measured (⇒ **Go to end of Questionnaire)**

Were you given your cholesterol results in writing?

☐ Yes
☐ No
☐ I did not want the result in writing
☐ Don't know

Do you have a target cholesterol level?

☐ Yes
☐ No
☐ Don't know

THANK YOU FOR COMPLETING THIS QUESTIONNAIRE - PLEASE BRING IT WITH YOU TO YOUR CLINIC VISIT AND HAND IT TO ONE OF THE STAFF

Patient ID .....

Date.....

EDINBURGH TYPE 2 DIABETES S

Year 4 Follow-Up

QUESTIONNAIRE

Please note: one of our research nurses will go over the questionnaire with you at the clinic and may ask a few additional questions

The information in this questionnaire is highly CONFIDENTIAL and is part of a medical research study

The information you give in this questionnaire will be treated as strictly confidential and will be available only to your own doctor and the study team. The results of the research will appear only in the form of general statistics from which it will be impossible to identify you as an individual.

Please complete the following:

SURNAME: ……..

FORENAMES: ……..

DATE: ……..

If you have any difficulties in answering some of the questions, you will have a chance to discuss these with a member of the study team.

Thank you for your co-operation in this study

YEAR 4 QUESTIONNAIRE

Many of the questions below may be familiar to you as they are similar to those you answered at our previous research clinics. However this time we are concentrating on what has happened to you during the 4 years since you attended our first research clinic. If you are unsure if a change in circumstances, a new diagnosis or test occurred during this period or not, please include it.

It is important to answer all the questions carefully.
Please take your time.

Personal History

Have you changed your address since we first saw you i.e. in the past 4 years?

Yes ☐ No ☐

If 'yes',
New address _____
_____
_____ Postcode _____
Telephone number _____

Have you changed your G.P. since we first saw you i.e. in the past 4 years?

Yes ☐ No ☐

If 'yes',
New GP name _____
Address _____
_____

Has your marital status changed since we first saw you i.e. in the past 4 years?

Yes ☐ No ☐

If 'yes', are you now:

☐ Married and/or living with long-term partner
☐ Single
☐ Widowed
☐ Divorced or separated

Current Employment Status

At the moment, what is the employment status of you and your spouse/ex-spouse or long-term partner?

You		Spouse/ex-partner/partner	
☐	Employed, full-time	☐	Employed, full-time
☐	Employed, part-time	☐	Employed, part-time
☐	Unemployed	☐	Unemployed
☐	Retired	☐	Retired
☐	Housewife (full-time)	☐	Housewife (full-time)
☐	Other *(please specify*)	☐	Other *(please specify)*

Medical History

Diabetes history

What treatment do you receive currently for your diabetes?

(i) Tablets Yes ☐ No ☐

If 'yes', please give name(s) _____

(ii) Insulin injections Yes ☐ No ☐

If 'yes',
give total number of units per day ☐☐☐units/day
what year did you start insulin ☐☐☐☐

In the past 4 years have you had an episode of low blood glucose (hypoglycaemia) when you have needed someone else to treat you eg. give sugary drink or glucagon?

Yes ☐ No ☐ Don't know ☐

If 'yes', how many times has this happened over the past 4 years?

☐ 1-2
☐ 3-4
☐ 5 or over

How many times has this happened over the past year?

☐ 1-2
☐ 3-4
☐ 5 or over

Are you on any of the following regular medical treatments from a doctor?

	Yes	No	Don't know
Aspirin	☐	☐	☐
Drugs for angina including spray	☐	☐	☐
Drugs to lower blood pressure	☐	☐	☐
Drugs to lower cholesterol	☐	☐	☐

Have you, to your knowledge, used any of the following medications over the last six months?

	Yes	No	Don't know
Amiodarone	☐	☐	☐
Isoniazid	☐	☐	☐
Methotrexate	☐	☐	☐
Allopurinol	☐	☐	☐
Tamoxifen	☐	☐	☐
Steroids (e.g. prednisolone, dexamethasone) – this includes oral steroids, steroid inhalers or steroid containing creams or eye drops	☐	☐	☐

If yes, please indicate when it was started and how long you were on it.

Medication	When started?	How long on it?
_____	_____	_____
_____	_____	_____
_____	_____	_____

Give names of all current medication if possible (including regular skin creams, eye drops, inhalers, tablets and injections which may or may not be repeat prescriptions):

		Office Use Only
1	_____	
2	_____	
3	_____	
4	_____	
5	_____	
6	_____	
7	_____	
8	_____	
9	_____	
10	_____	

Vascular and Liver Disease

We are interested in any diagnoses of vascular disease or liver conditions which you have had over the past 4 years. If you have been told that you have had one or more of the events mentioned in the next few questions (numbers 0 to 0), but can't remember whether the event occurred in the past 4 years or not, please include it anyway and tick 'yes' to the relevant question.

Have you experienced either of the following since we first saw you in the research clinic i.e. during the past 4 years?

		Yes	No	Don't know
(i)	Heart attack (coronary thrombosis, myocardial infarction)	☐	☐	☐
(ii)	Stroke	☐	☐	☐

If you have answered 'yes' to either of the above, please give the year in which the event occurred (as near as you can remember) and the name of the hospital/GP surgery where you were/are treated for the condition

Event/condition	Year of event/diagnosis	Hospital/GP surgery where treated

Have you been told <u>by a doctor</u> that you have developed any of the following for the first time in the past 4 years?

		Yes	No	Don't know
(i)	Angina	☐	☐	☐
(ii)	Hardening of the arteries in the legs	☐	☐	☐
(iii)	High blood pressure	☐	☐	☐

If you have answered 'yes' to any of the above, please give the year in which the condition was diagnosed (as near as you can remember) and the name of the hospital/GP surgery where you were/are treated for the condition

Event/condition	Year of event/diagnosis	Hospital/GP surgery where treated

During the past 4 years have you undergone any of the following procedures/operations?

		Yes	No	Don't know
(i)	An operation or balloon treatment to relieve a blockage in the arteries of your heart (coronary-by-pass or angioplasty)	☐	☐	☐
(ii)	An operation or balloon treatment to relieve a blockage in the arteries of your leg(s), other than for varicose veins	☐	☐	☐
(iii)	Surgery to remove toes or leg (above or below the knee)	☐	☐	☐
(iv)	An operation or balloon treatment to relieve a blockage in the arteries of your neck (carotid surgery, angioplasty or stenting)	☐	☐	☐

If you have answered 'yes' to any of the above, please give the year in which the procedure was performed and the name of the hospital you attended

Procedure/operation	Year performed	Hospital attended

Have you been told by a doctor that you have developed any of the following for the first time in the past 4 years?

		Yes	No	Don't know
(i)	Hepatitis	☐	☐	☐
(ii)	Cirrhosis of the liver	☐	☐	☐
(iii)	Any other disease/medical condition affecting the liver	☐	☐	☐

If you have answered 'yes' to any of the above, please give the name of the condition, the year in which it was diagnosed (as near as you can remember) and the name of the hospital where you were/are treated for the condition

Name of condition	Year of diagnosis	Hospital where treated

Other conditions

Although we are mainly interested in vascular diseases and liver disease, we also need to know about any other conditions which you may have developed over the past 4 years as this may affect some of the tests we do.

Have you been told by a doctor that you may have developed any of the following for the first time over the past 4 years?

		Yes	No	Don't know
(i)	Disease affecting the joints	☐	☐	☐
(ii)	Disease of the thyroid gland	☐	☐	☐
(iii)	Any other medical condition not mentioned elsewhere in the questionnaire	☐	☐	☐

If you have answered 'yes' to any of the above, please give the name of the condition and the year in which it was diagnosed (as near as you can remember).

Name of condition	Year of diagnosis	Name of condition	Year of diagnosis
1.		5.	
2.		6.	
3.		7.	
4.		8.	

Birth weight and menstrual history

We are interested in looking at the role of birth weight and reproductive history in the subsequent development of disease. Please answer the following questions as far as you can remember.

Do you know roughly what your birth weight was?

Yes ☐ No ☐

If 'yes', what was it?

☐☐ lbs ☐☐ oz

How many children have you had?

☐☐ children

WOMEN only (MEN please go to Question 0)

How old were you when you started your periods?

☐☐ years Don't know ☐

How old were you when your periods stopped?

☐☐ years Don't know ☐

The remaining questions in this questionnaire are the same as those we asked you in previous questionnaires. Although you may find that you are giving very similar answers to those you gave previously, please complete all of these as accurately as possible, as they will enable us to work out if anything has changed since we first saw you.

Alcohol

Current alcohol intake

Think back carefully over the last seven days. Please write in each column the exact number of alcoholic drinks you consumed on each day during the past week. If none consumed write '0' in the boxes.

Try to remember where and who you were with on each day. This may help you remember what you had to drink.

	Pints of beer, lager, cider etc	Single glasses of whisky, vodka, gin etc	Single glasses of martini, wine, sherry etc
Monday	☐	☐	☐
Tuesday	☐	☐	☐
Wednesday	☐	☐	☐
Thursday	☐	☐	☐
Friday	☐	☐	☐
Saturday	☐	☐	☐
Sunday	☐	☐	☐

Would you say that last week was fairly typical of what you usually have to drink in a week?

Yes ☐ No ☐

If last week was not typical, would you normally drink more or less in a week?

More ☐ Less ☐

Alcohol intake over the past year

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).

☐ Never
☐ Monthly or less
☐ 2 to 4 times a month
☐ 2 to 3 times a week
☐ 4 to 5 times a week
☐ 6 or more times a week

How many drinks did you have on a typical day when you were drinking in the past year?

- ☐ 0 drinks
- ☐ 1 to 2 drinks
- ☐ 3 to 4 drinks
- ☐ 5 to 6 drinks
- ☐ 7 to 9 drinks
- ☐ 10 or more drinks

How often did you have 6 or more drinks on one occasion in the past year?

- ☐ Never
- ☐ Less than monthly
- ☐ Monthly
- ☐ Weekly
- ☐ Daily or almost daily

Have you or your doctor ever considered that you suffer/have in the past suffered from an alcohol problem/excessive drinking?

Yes ☐ No ☐

Smoking

Do you smoke cigarettes at present?

Yes ☐ No ☐

If no, please go to Question 0

If ‘yes’, how many cigarettes do you usually smoke now?

☐☐ per day

How old were you when you started smoking cigarettes?

☐☐ years

How many cigarettes have you smoked on average per day during the period you have smoked

☐☐ cigarettes per day

Now please go to Question 0

Have you ever smoked cigarettes regularly?

Yes ☐ No ☐

If no, please go to Question 0

If ‘yes’, how many cigarettes did you smoke on average when you were a smoker?

☐☐ per day

How old were you when you started smoking cigarettes?

☐☐ years

How long is it since you finally gave up?

☐☐ years and ☐☐ months

Chest Pain

Do you ever get pain or discomfort in your chest?

Yes ☐ No ☐

If no, please go to Question 0

Do you get this pain or discomfort when you walk uphill or hurry?

Yes ☐ No ☐

If no, please go to question 0

Do you get it when you walk at an ordinary pace on the level?

Yes ☐ No ☐

When you get any pain or discomfort in your chest what do you do? *(Tick one only)*

Stop ☐ Slow down ☐ Continue at the same pace ☐

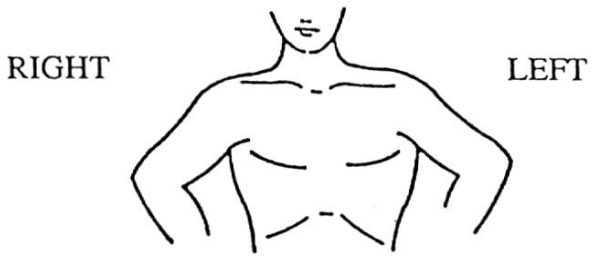
Does it go away when you stand still or sit down?

Yes ☐ No ☐

If yes, how soon? *(Tick one only)*

10 minutes or less ☐ More than 10 minutes ☐

Where do you get this pain or discomfort? Mark the place(s) with an 'X' on the diagram



Have you ever had a severe pain across the front of your chest lasting for half an hour?

Yes ☐ No ☐

If 'yes', what was the cause? _____

Leg Pain

Do you get a pain or discomfort in your leg(s) when you walk?

Yes ☐ No ☐ I am unable to walk ☐

If no, you do not need to complete anymore of the questionnaire

Please answer the following questions about your leg pain

		Yes	No
(i)	Does this pain ever begin when you are standing still or sitting?	☐	☐
(ii)	Do you get it if you walk uphill or hurry?	☐	☐
(iii)	Do you get it when you walk at an ordinary pace on the level?	☐	☐
(iv)	Does the pain ever disappear while you are still walking?	☐	☐

(v) What do you do if you get it when you are walking?

Stop ☐ Slow down ☐ Continue at the same pace ☐

(vi) What happens to it if you stand still?

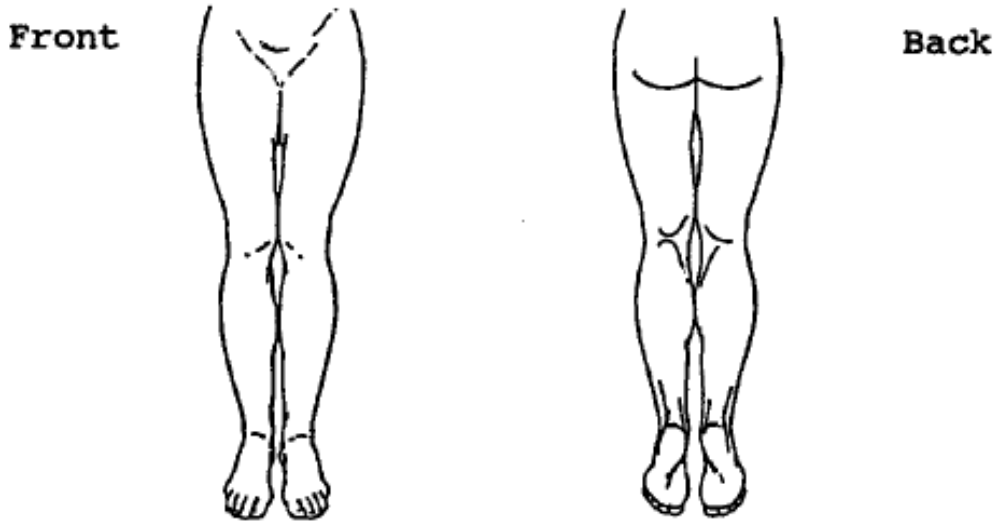
☐ Usually continues for more than 10 minutes
☐ Usually disappears in 10 minutes or less

(vii) Where do you get this pain or discomfort?

Do you get this pain in your calf (or calves)?

Yes ☐ No ☐

Please mark the place(s) where you get the pain with 'X' on the diagram below



Thank you for completing this questionnaire – please bring it with you to your appointment at the Wellcome Trust Clinical Research Facility

Appendix I Variables measured in the ET2DS

TableI-1 Variables measured in the ET2DS

Variable	*Baseline*	*Year 1*	*Year 4*
Demographics			
Age	x	-	-
Sex	x	-	-
Marital status	x	-	-
Occupation	x	-	-
SIMD	x	-	-
Ethnicity	x	-	-
Education	x	-	-
Employment	x	-	-
Diabetes history			
Year of diagnosis	x	-	-
Current treatment	x	x	x
Hypo episodes	x	x (up to 6 months prior)	x
Fasting BG	x	x	x
HBA1c	x	x	x
Miscellaneous			
Medications	x	x	x
History of joint conditions	-	x	
Sleep apnoea Q's	-	x	-
Use of HC services	-	x	-
Intra-abdominal pathology	-	x	
Cognitive and mood tests			
MHVS	x	-	x
DST	x	-	x
LNS	x	-	x
MR	x	-	x
Faces	x	-	x
Logical Memory	x	-	x
TMT-B	x	-	x
Verbal fluency	x	-	x
MMSE	x	-	x
HAD	x	-	x
Reaction time	-	-	x

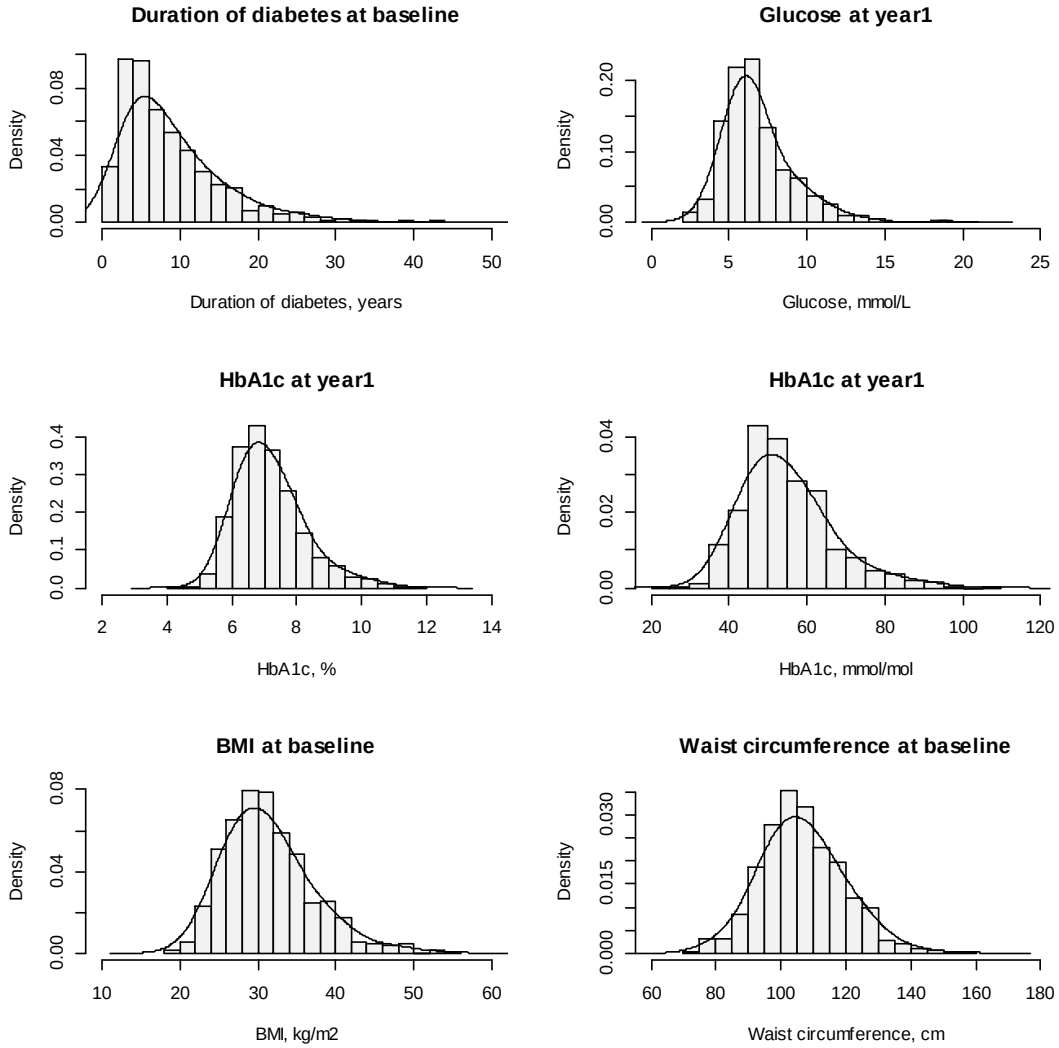
Variable	*Baseline*	*Year 1*	*Year 4*
Retinopathy			
ETDRS grading	x		
Retinal vessel Mx	x		
Cardiovascular disease/risk factors			
CV events			
IHD	x	x	x
Cerebrovascular disease	x	x	x
PAD	x	x	x
ECG	x	-	x
% body fat	x	-	-
BMI	x	-	x
WHR	x	-	x
sBP, dBP	x	x	x
ABI	x	-	x
Smoking history	x	-	x
Alcohol intake	x	x	x
Neurothesiometry	x	-	-
cIMT/plaque	-	x	x
Pulse wave analysis	-	x	-
Pulse wave velocity	-	x	-
Clot structure			
Final turbidity (clot density)	-	x	
Clot formation time	-	x	
Fibrinolysis time	-	x	
'Psychological'			
Stress	x	-	-
Personality etc.	x	-	-
Markers			
tChol	x	x	x
HDL	x	x	x
TGs	-	x	x
Apolipoprotein AI	-	x	x
Apolipoprotein B	-	x	x
Free fatty acids	-	x	x
LFTs	x	x	x
FBC	x	x	x
HA	x	-	-

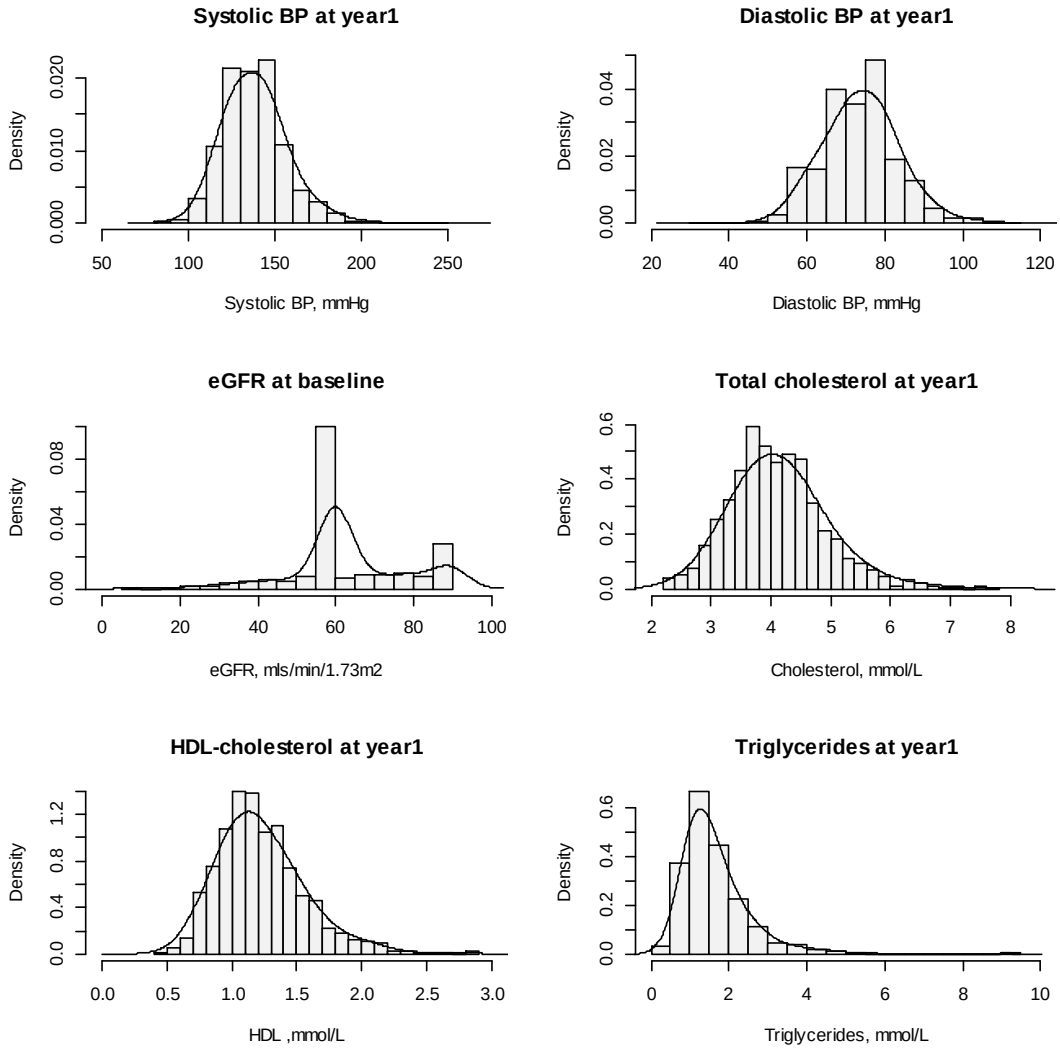
Variable	*Baseline*	*Year 1*	*Year 4*
Fibrinogen	x	-	-
TNF alpha	x	-	-
IL-6	x	-	-
CRP	x	-	-
PV	x	-	-
Leptin	x		
ELF panel	x (stored plasma)	x (stored plasma)	
Creatinine	x	-	x
Urinary albumin	x	-	
Urinary creatinine	x	-	
ACR	x	-	
Cortisol	x (fasting am)	-	x (timed)
Uric acid	-	x	
TFTs			
Free T3	-	x	
Free T4	-	x	
TSH	-	x	
Total T3	-	x	
Total T4	-	x	
Sex hormones			
Total testosterone	-	x	
Free testosterone	-	x	
Bioavailable testosterone	-	x	
SHBG	-	x	
Complement C3	-	x	
Asymmetric dimethylarginine	-	x	
Symmetric dimethylarginine	-	x	
L-arginine	-	x	
NT proBNP	x		
Oxidative stress		(x)	
Glycans	x (if funded)		
Liver disease			
History of liver disease/investigations	x	x	x
Steatosis (USS)	-	x	x
MRS (subgroup)	-	x	
TE (Fibroscan)	-	-	x

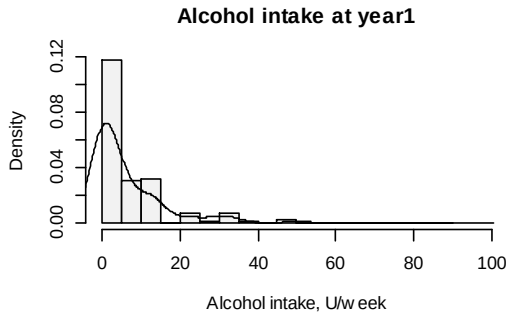
Variable	*Baseline*	*Year 1*	*Year 4*
MRE (subgroup)	-	-	(x)
Stored samples			
Plasma/serum	x	x	x
Urine	x	-	x
DNA	x	-	-
WBCs	x	-	-

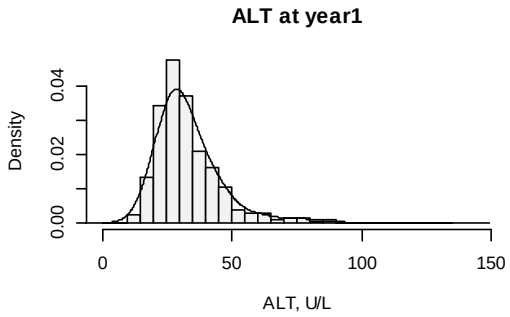
Appendix J Distributions of variables in the ET2DS

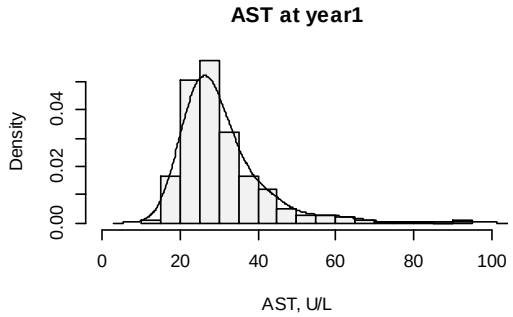
Figure J-1 Histograms showing the distributions of markers of liver injury in the whole study population.

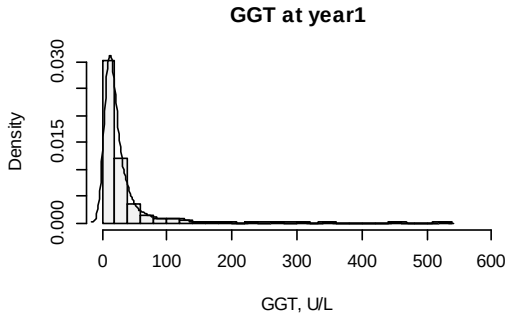


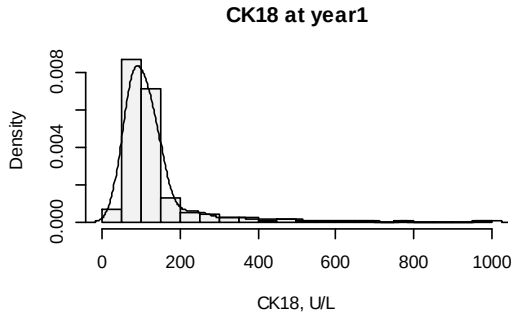

Systolic BP at year1
Density
0.000
0.010
0.020
50
100
150
200
250
Systolic BP, mmHg
Diastolic BP at year1
Density
0.00
0.02
0.04
20
40
60
80
100
120
Diastolic BP, mmHg
eGFR at baseline
Density
0.00
0.04
0.08
0
20
40
60
80
100
eGFR, mls/min/1.73m2
Total cholesterol at year1
Density
0.0
0.2
0.4
0.6
2
3
4
5
6
7
8
Cholesterol, mmol/L
HDL-cholesterol at year1
Density
0.0
0.4
0.8
1.2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
HDL ,mmol/L
Triglycerides at year1
Density
0.0
0.2
0.4
0.6
0
2
4
6
8
10
Triglycerides, mmol/L

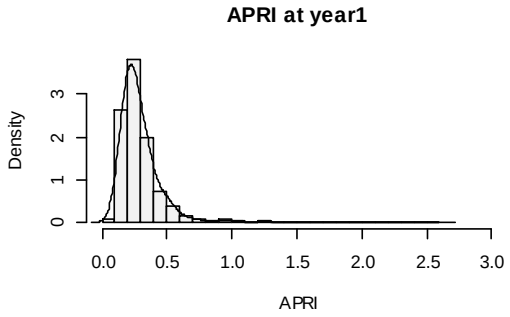

Alcohol intake at year1
Density
0.00
0.04
0.08
0.12
0
20
40
60
80
100
Alcohol intake, U/week

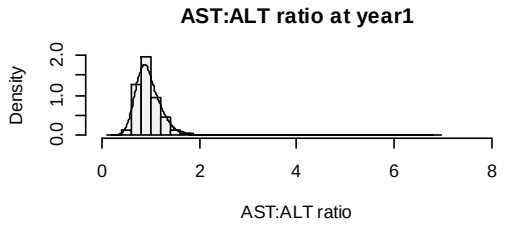

ALT at year1
Density
0.00
0.02
0.04
0
50
100
150
ALT, U/L

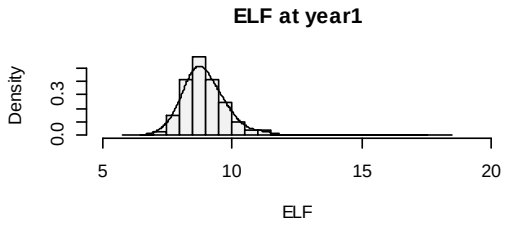

AST at year1
Density
0.00
0.02
0.04
0
20
40
60
80
100
AST, U/L

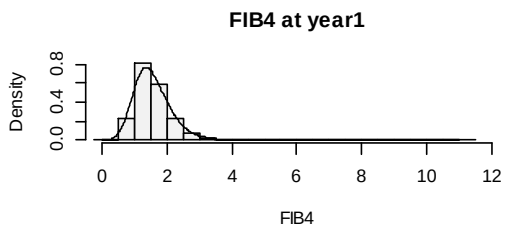

GGT at year1
Density
0.000
0.015
0.030
0
100
200
300
400
500
600
GGT, U/L

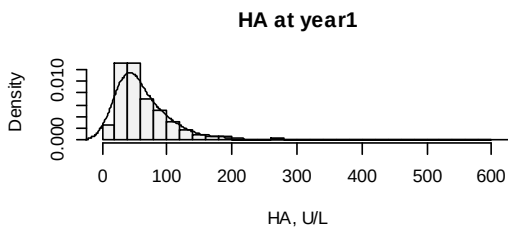

CK18 at year1
Density
0.000
0.004
0.008
0
200
400
600
800
1000
CK18, U/L

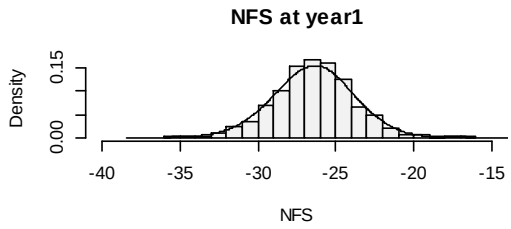

APRI at year1
Density
0
1
2
3
0.0
0.5
1.0
1.5
2.0
2.5
3.0
APRI

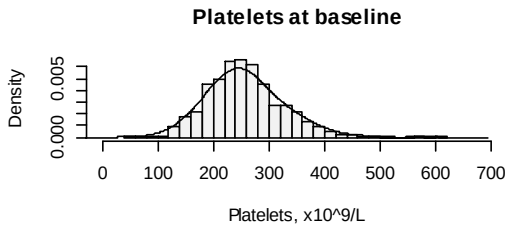

AST:ALT ratio at year1
Density
0.0
1.0
2.0
0
2
4
6
8
AST:ALT ratio

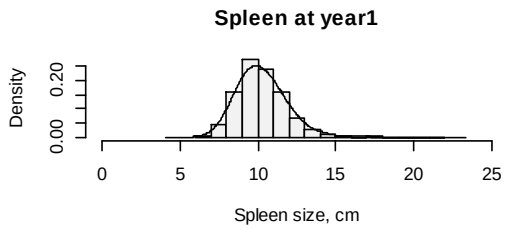

ELF at year1
Density
0.0
0.3
5
10
15
20
ELF


FIB4 at year1
Density
0.0
0.4
0.8
0
2
4
6
8
10
12
FIB4


HA at year1
Density
0.000
0.010
0
100
200
300
400
500
600
HA, U/L

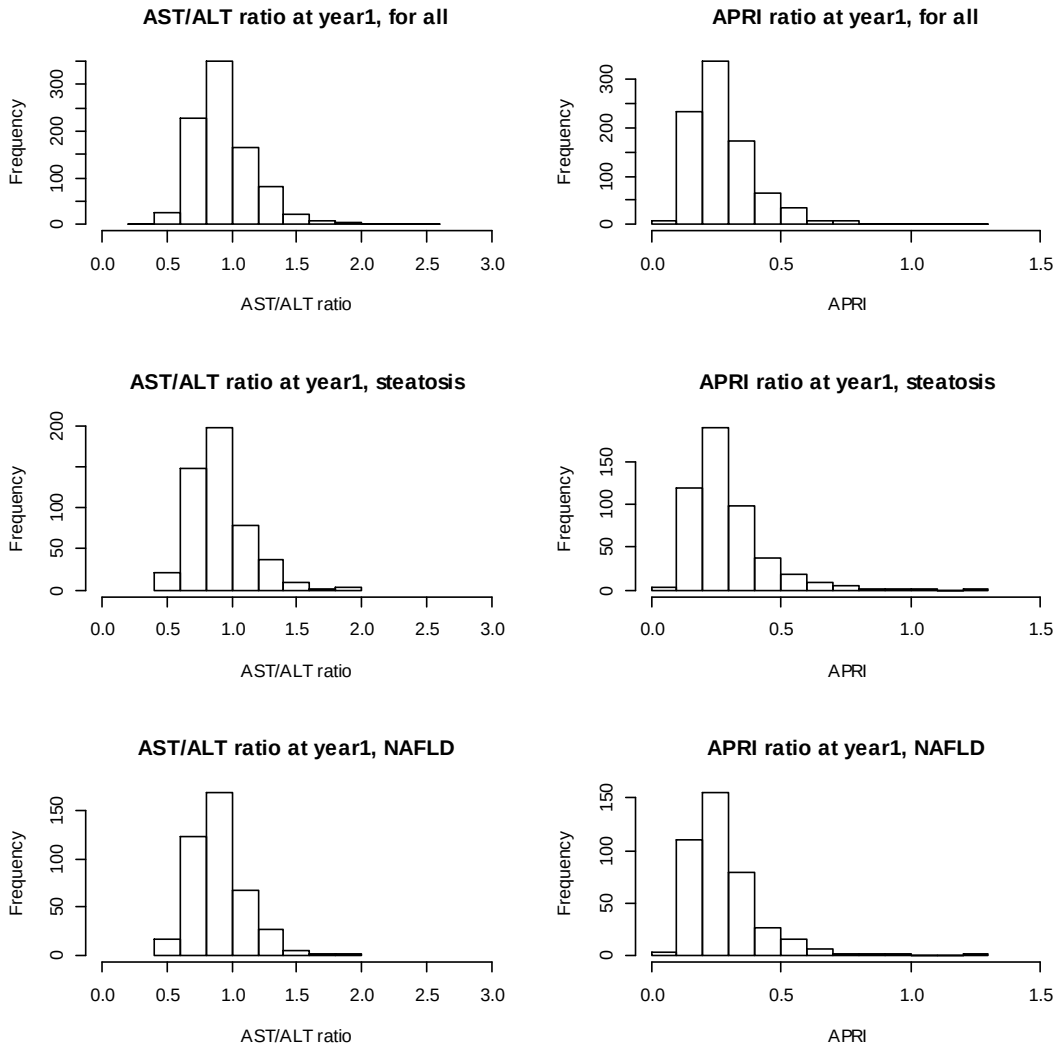

NFS at year1
Density
0.00
0.15
-40
-35
-30
-25
-20
-15
NFS

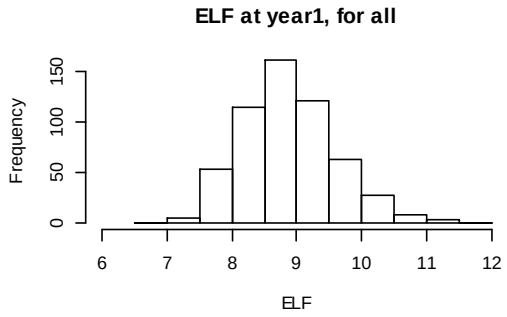

Platelets at baseline
Density
0.000
0.005
0
100
200
300
400
500
600
700
Platelets, x10^9/L

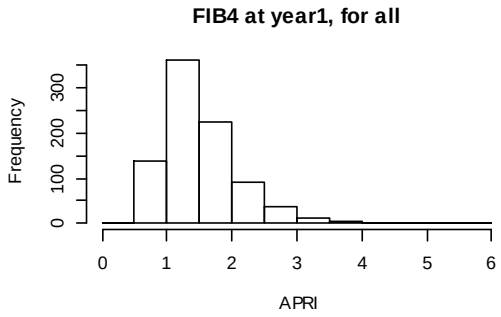

Spleen at year1
Density
0.00
0.20
0
5
10
15
20
25
Spleen size, cm

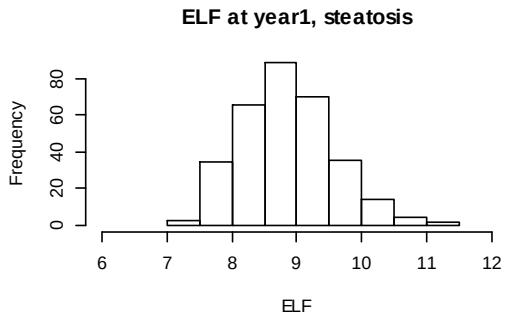
Appendix K Liver injury marker distributions (histograms)

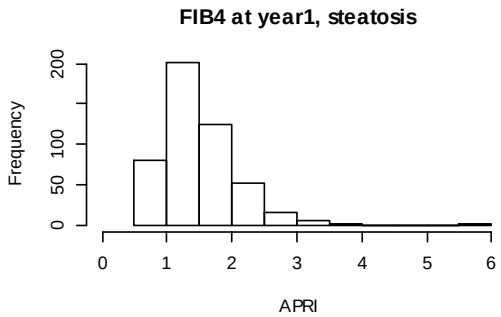
Figure K-1 Histograms showing the distributions of markers of liver injury in the whole study population, those with hepatic steatosis and those with NAFLD.

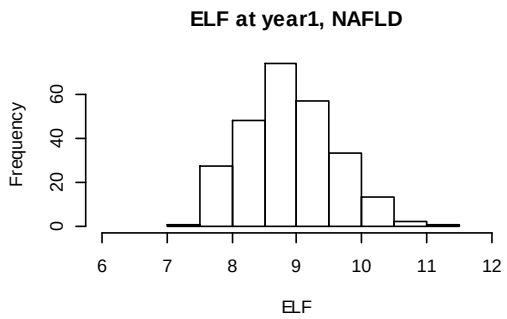


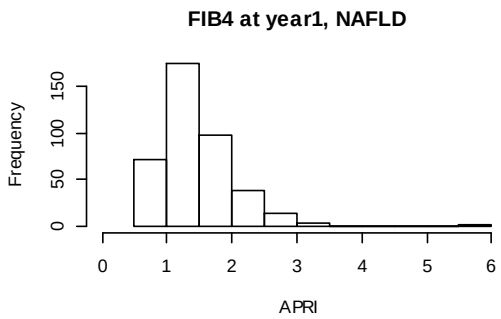

ELF at year1, for all
Frequency
0
50
100
150
6
7
8
9
10
11
12
ELF

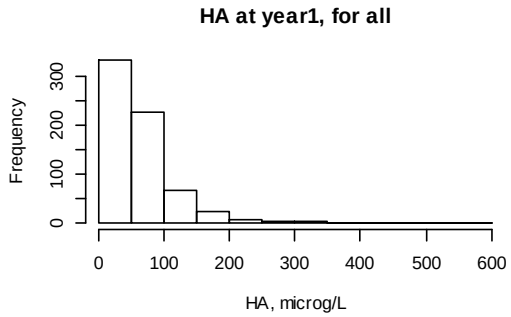

FIB4 at year1, for all
Frequency
0
100
200
300
0
1
2
3
4
5
6
APRI

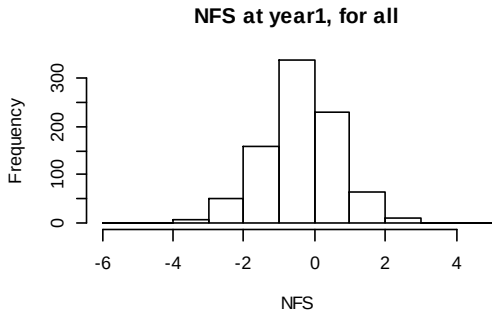

ELF at year1, steatosis
Frequency
0
20
40
60
80
6
7
8
9
10
11
12
ELF

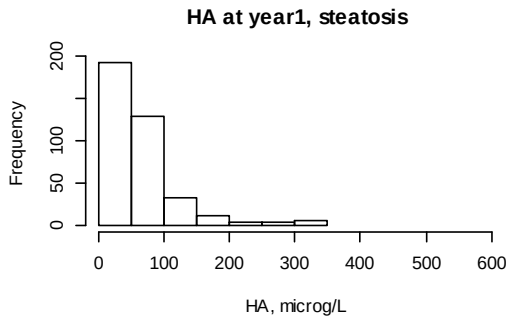

FIB4 at year1, steatosis
Frequency
0
50
100
200
0
1
2
3
4
5
6
APRI

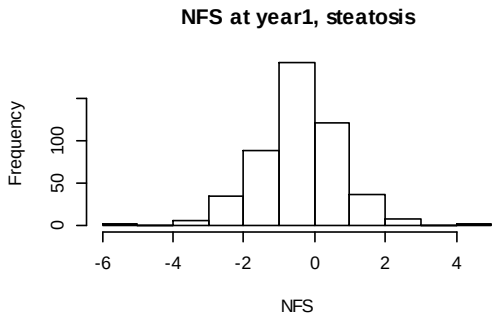

ELF at year1, NAFLD
Frequency
0
20
40
60
6
7
8
9
10
11
12
ELF

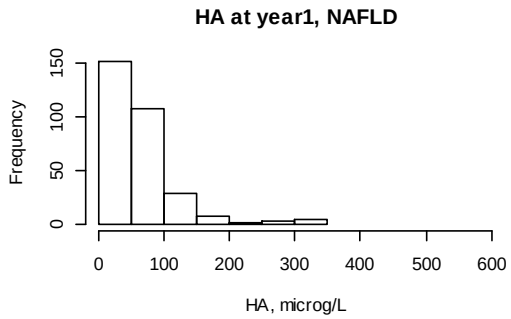

FIB4 at year1, NAFLD
Frequency
0
50
100
150
0
1
2
3
4
5
6
APRI

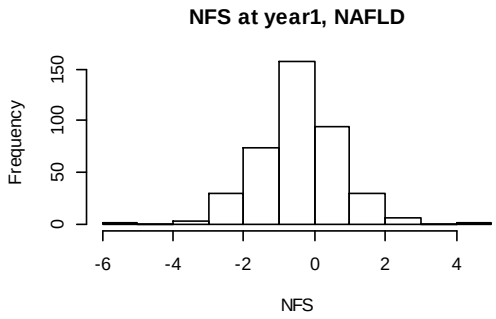

HA at year1, for all
Frequency
0
100
200
300
0
100
200
300
400
500
600
HA, microg/L

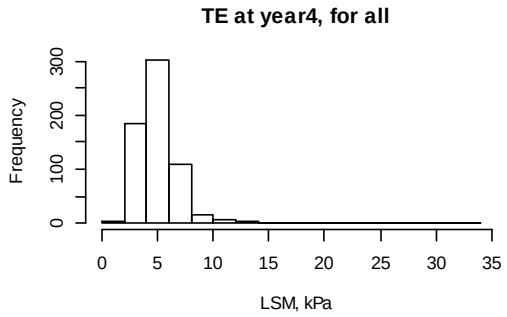

NFS at year1, for all
Frequency
0
100
200
300
-6
-4
-2
0
2
4
NFS

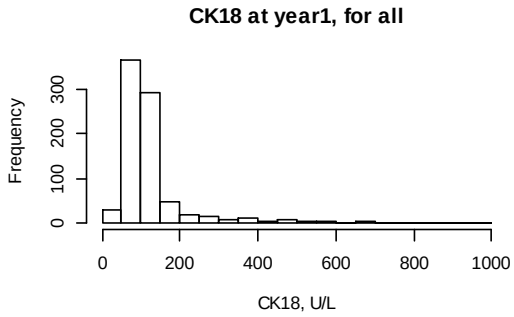

HA at year1, steatosis
Frequency
0
50
100
200
0
100
200
300
400
500
600
HA, microg/L

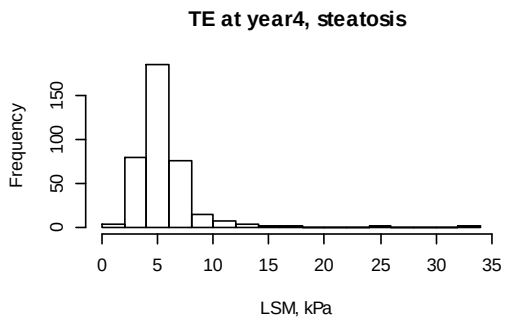

NFS at year1, steatosis
Frequency
0
50
100
-6
-4
-2
0
2
4
NFS

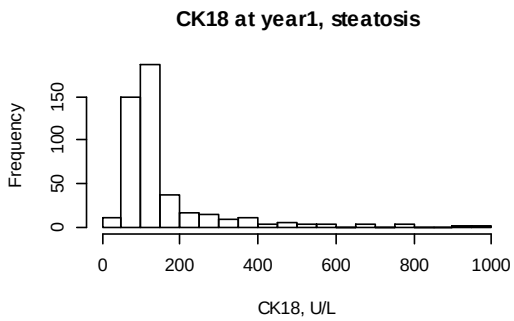

HA at year1, NAFLD
Frequency
0
50
100
150
0
100
200
300
400
500
600
HA, microg/L

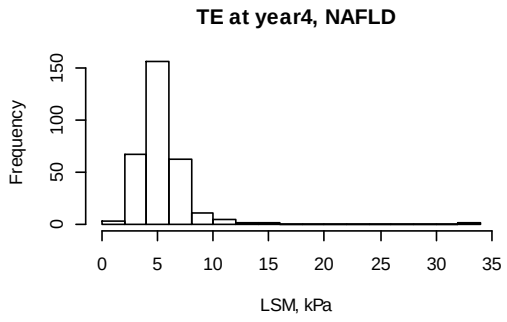

NFS at year1, NAFLD
Frequency
0
50
100
150
-6
-4
-2
0
2
4
NFS

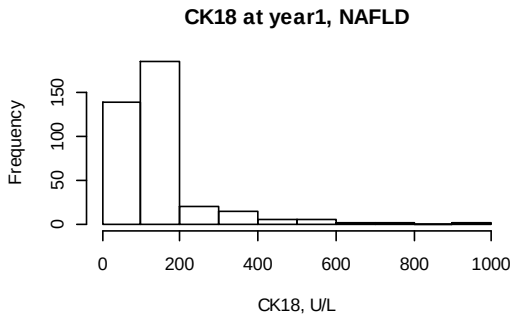

TE at year4, for all
Frequency
0
100
200
300
0
5
10
15
20
25
30
35
LSM, kPa


CK18 at year1, for all
Frequency
0
100
200
300
0
200
400
600
800
1000
CK18, U/L


TE at year4, steatosis
Frequency
0
50
100
150
0
5
10
15
20
25
30
35
LSM, kPa

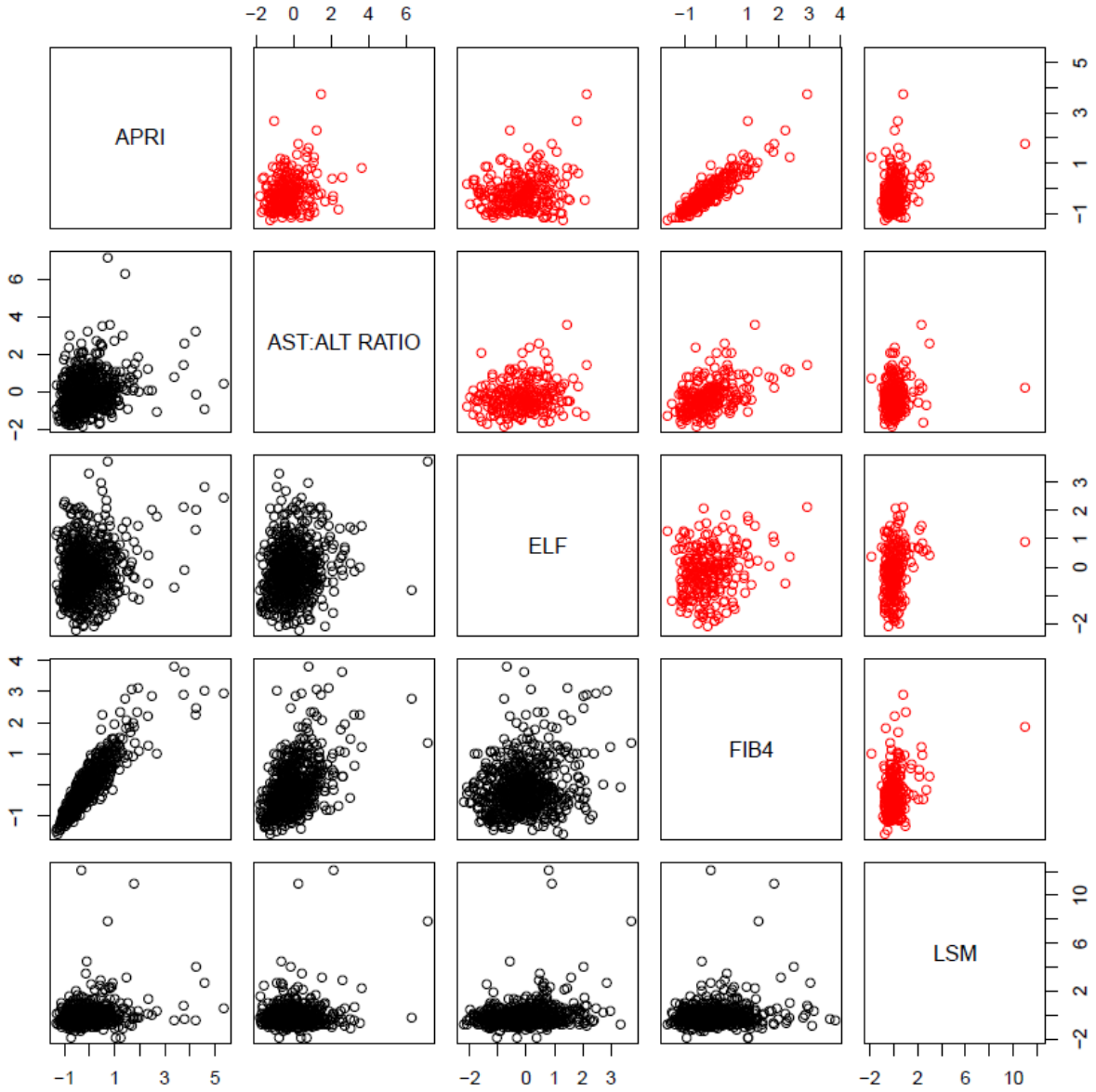

CK18 at year1, steatosis
Frequency
0
50
100
150
0
200
400
600
800
1000
CK18, U/L


TE at year4, NAFLD
Frequency
0
50
100
150
0
5
10
15
20
25
30
35
LSM, kPa


CK18 at year1, NAFLD
Frequency
0
50
100
150
0
200
400
600
800
1000
CK18, U/L

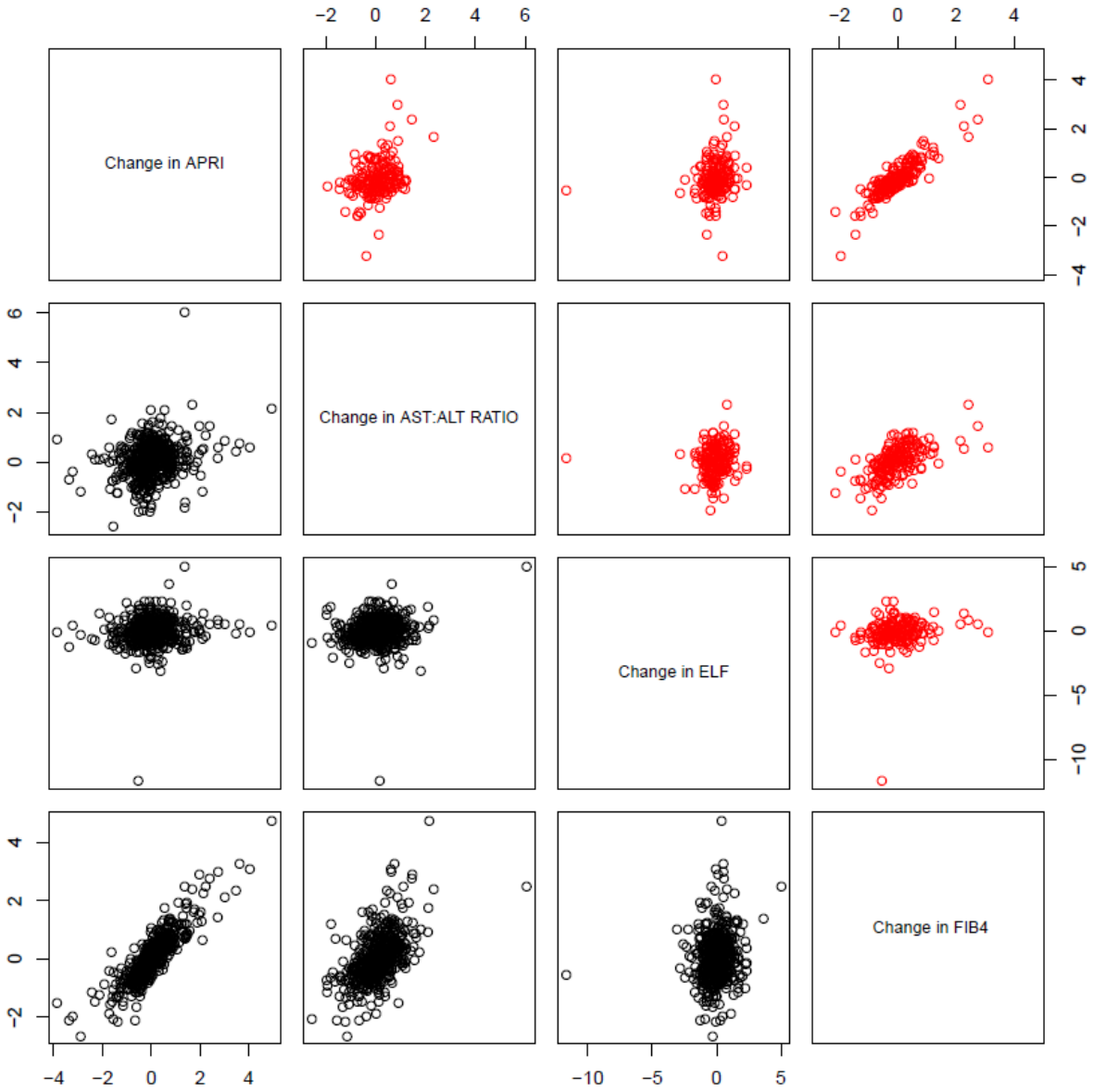
Appendix L Liver injury marker agreement (scatter plots and 2x2 tables)

Figure L-1 Scatter plots of markers correlations



Lower panel (black) = all patients
Upper panel (red) = NAFLD patients

Figure L-2 Scatter plots of change in marker correlations



Lower panel (black) = all patients
Upper panel (red) = NAFLD patients

Table L-1 Comparison of surrogate markers of advanced portal hypertension between the top 5% and lower 95% of all subjects. Values are mean (sd).

		Spleen diameter (cm)	*p*	Platelet count (x10^9/L	*p*
APRI	Top 5%	11.8 (1.8)	***<0.001***	154.5 (36.9)	***<0.001***
	Bottom 95%	10.3 (1.5)		233.7 (65.7)	
AST:ALT ratio	Top 5%	10.6 (2.0)	*0.410*	231.0 (67.5)	*0.911*
	Bottom 95%	10.4 (1.6)		229.7 (66.8)	
ELF	Top 5%	11.3 (2.1)	***0.008***	212.3 (85.2)	*0.199*
	Bottom 95%	10.4 (1.5)		230.7 (65.7)	
FIB4	Top 5%	11.7 (1.8)	***<0.001***	149.7 (37.0)	***<0.001***
	Bottom 95%	10.3 (1.5)		233.9 (65.4)	
LSM	Top 5%	11.6 (1.6)	***<0.001***	203.3 (51.5)	***0.019***
	Bottom 95%	10.3 (1.5)		232.1 (66.9)	

ALT alanine aminotransferase; **APRI** aspartate to platelet ratio index; **AST** aspartate aminotransferase; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; **LSM** liver stiffness measure.

Table L-2 Comparison of surrogate markers of advanced portal hypertension between the top 10% and lower 90% of subjects with non-alcoholic fatty liver. Values are mean (sd).

		Spleen diameter (cm)	*p*	Platelet count (x10^9/L	*p*
APRI	Top 10%	11.1 (1.6)	***0.008***	170.5 (39.0)	***<0.001***
	Bottom 90%	10.3 (1.5)		246.5 (65.6)	
AST:ALT ratio	Top 10%	10.4 (1.7)	*0.901*	231.3 (65.8)	*0.524*
	Bottom 90%	10.4 (1.5)		239.8 (66.9)	
ELF	Top 10%	10.9 (1.9)	***0.044***	248.4 (109.1)	*0.621*
	Bottom 90%	10.3 (1.5)		137.9 (60.3)	
FIB4	Top 10%	11.4 (1.7)	***<0.001***	163.3 (28.6)	***<0.001***
	Bottom 90%	10.3 (1.4)		247.3 (64.2)	
LSM	Top 10%	10.8 (1.3)	*0.116*	236.6 (74.9)	*0.853*
	Bottom 90%	10.3 (1.5)		239.3 (65.2)	

ALT alanine aminotransferase; **APRI** aspartate to platelet ratio index; **AST** aspartate aminotransferase; **ELF** Enhanced Liver Fibrosis panel; **FIB4** Fibrosis-4 Index; **LSM** liver stiffness measure.

Figure L-3 2x2 tables for all markers, top 5%

		APRI		
		Fibrosis	No fib	Total
AST/ALT ratio	Fibrosis	7	31	38
	No fib	31	698	729
	Total	38	729	767

		ELF		
		Fibrosis	No fib	Total
AST/ALT ratio	Fibrosis	7	31	38
	No fib	31	698	729
	Total	38	729	767

		FIB4		
		Fibrosis	No fib	Total
AST/ALT ratio	Fibrosis	13	25	38
	No fib	25	704	729
	Total	38	729	767

		LSM		
		Fibrosis	No fib	Total
AST/ALT ratio	Fibrosis	3	29	32
	No fib	28	588	616
	Total	31	617	648

		ELF		
		Fibrosis	No fib	Total
APRI	Fibrosis	12	26	38
	No fib	26	703	729
	Total	38	729	767

		FIB4		
		Fibrosis	No fib	Total
APRI	Fibrosis	29	9	38
	No fib	9	720	729
	Total	38	729	767

		LSM		
		Fibrosis	No fib	Total
APRI	Fibrosis	4	28	32
	No fib	27	589	616
	Total	31	617	648

		FIB4		
		Fibrosis	No fib	Total
ELF	Fibrosis	13	25	38
	No fib	25	704	729
	Total	38	729	767

		LSM		
		Fibrosis	No fib	Total
ELF	Fibrosis	5	27	32
	No fib	26	590	616
	Total	31	617	648

		LSM		
		Fibrosis	No fib	Total
FIB4	Fibrosis	4	28	32
	No fibrosis	27	589	616
	Total	31	617	648

		APRI		
		Fibrosis	No fib	Total
AST/ALT ratio	Fibrosis	8	20	28
	No fib	20	234	254
	Total	28	254	282

		ELF		
		Fibrosis	No fib	Total
AST/ALT ratio	Fibrosis	4	24	28
	No fib	24	230	254
	Total	28	254	282

		FIB4		
		Fibrosis	No fib	Total
AST/ALT ratio	Fibrosis	10	18	28
	No fib	18	236	254
	Total	28	254	282

		LSM		
		Fibrosis	No fib	Total
AST/ALT ratio	Fibrosis	7	17	24
	No fib	17	206	223
	Total	24	223	247

		ELF		

		FIB4		

APRI

	Fibrosis	No fib	Total
Fibrosis	8	20	28
No fib	20	234	254
Total	28	254	282

APRI

	Fibrosis	No fib	Total
Fibrosis	19	9	28
No fib	9	245	254
Total	28	254	282

APRI

	LSM		
	Fibrosis	No fibrosis	Total
Fibrosis	5	19	24
No fib	19	204	223
Total	24	223	247

ELF

	FIB4		
	Fibrosis	No fib	Total
Fibrosis	9	19	28
No fib	19	235	254
Total	28	254	282

ELF

	LSM		
	Fibrosis	No fib	Total
Fibrosis	6	18	24
No fib	18	205	223
Total	24	223	247

FIB4

	LSM		
	Fibrosis	No fib	Total
Fibrosis	6	18	24
No fib	18	205	223
Total	24	223	247

Appendix M Markers of chronic liver disease and cardiovascular disease

Table M-1 Multivariable association between liver markers and incident cardiovascular disease events (all subjects). Values are hazard ratios (95%CI)

	Model 1	*p*	*Model 2*	*p*	*Model 3*	*p*
ALT, U/L	0.99 (0.98,1.01)	0.379	0.99 (0.98,1.01)	0.262	0.99 (0.97,1.00)	0.140
AST, U/L	1.00 (0.99,1.02)	0.674	1.00 (0.98,1.02)	0.888	1.00 (0.98,1.02)	0.794
GGT, log_2[a]	1.28 (1.10,1.49)	0.001	1.26 (1.08,1.47)	0.003	1.18 (1.00,1.39)	0.045
Steatosis, % yes[b]	1.24 (0.75,2.04)	0.399	1.32 (0.80,2.17)	0.282	1.31 (0.78,2.20)	0.307
CK18, log_2[a, b]	1.15 (0.84,1.56)	0.380	1.20 (0.88,1.64)	0.254	1.07 (0.78,1.46)	0.687
APRI, log_2[a]	0.97 (0.69,1.36)	0.873	0.81 (0.56,1.17)	0.263	0.78 (0.54,1.13)	0.193
AST:ALT ratio	2.90 (0.92,9.16)	0.069	2.67 (0.86,8.33)	0.090	2.85 (0.90,8.98)	0.074
ELF score[c]	1.20 (0.98,1.48)	0.082	1.23 (0.97,1.56)	0.095	1.15 (0.88,1.50)	0.304
FIB4	1.15 (0.82,1.62)	0.421	0.94 (0.63,1.39)	0.747	0.91 (0.61,1.34)	0.621
NFS	0.97 (0.90, 1.06)	0.542	0.98 (0.90,1.07)	0.642	0.96 (0.88,1.04)	0.320
Platelets, $x10^9$/L	1.00 (1.00,1.00)	0.596	1.00 (1.00,1.01)	0.071	1.00 (1.00,1.01)	0.101

[a] APRI, CK18 and GGT analysed on the Log_2 scale for linearization, therefore odds ratios relate to a doubling of the marker; [b] Incident CVD n=30/561; [c] incident CVD n=24/444.

Model 1 – Unadjusted; Model 2 - Adjusted for age and sex; Model 3 - Adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, body mass index, systolic blood pressure, diastolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and estimated glomerular filtration rate. Incident analysis additionally adjusted for prevalence cardiovascular disease at baseline.

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

Table M-2 Multivariable association between liver markers and incident coronary artery disease events (all subjects). Values are hazard ratios (95%CI)

	Model 1	*p*	*Model 2*	*p*	*Model 3*	*p*
ALT, U/L	1.00 (0.98,1.02)	0.976	1.00 (0.98,1.02)	0.911	1.00 (0.98,1.02)	0.742
AST, U/L	1.02 (1.00,1.04)	0.148	1.02 (0.99,1.04)	0.151	1.01 (0.99,1.03)	0.316
GGT, log_2[a]	1.31 (1.10,1.58)	0.003	1.33 (1.11,1.59)	0.002	1.24 (1.02,1.51)	0.032
Steatosis, % yes[b]	1.62 (0.86,3.05)	0.133	1.70 (0.90,3.20)	0.103	1.12 (0.82,3.04)	0.175
CK18, log_2[a, b]	1.15 (0.81,1.65)	0.440	1.20 (0.83,1.73)	0.328	1.08 (0.74,1.57)	0.687
APRI, log_2[a]	1.06 (0.70,1.60)	0.775	0.98 (0.63,1.53)	0.932	0.93 (0.60,1.45)	0.751
AST:ALT ratio	3.95 (1.03,15.17)	0.045	3.39 (0.87,13.28)	0.080	3.72 (0.96,14.53)	0.058
ELF score[c]	1.18 (0.89,1.55)	0.251	1.13 (0.81,1.57)	0.474	1.019 (0.68,1.49)	0.963
FIB4	1.31 (0.89,1.92)	0.167	1.16 (0.75,1.79)	0.504	1.11 (0.72,1.71)	0.628
NFS	1.00 (0.90,1.10)	0.934	0.99 (0.89,1.10)	0.859	0.97 (0.87,1.07)	0.535
Platelets, x10^9/L	1.00 (1.00,1.0)	0.359	1.00 (1.00,1.01)	0.114	1.00 (1.00,1.01)	0.132

[a] APRI, CK18 and GGT analysed on the Log_2 scale for linearization, therefore odds ratios relate to a doubling of the marker; [b] incident CAD n=16/561; [c] incident CAD n=13/444.

Model 1 – Unadjusted; Model 2 - Adjusted for age and sex; Model 3 - Adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, body mass index, systolic blood pressure, diastolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and estimated glomerular filtration rate. Incident analysis additionally adjusted for prevalence cardiovascular disease at baseline.

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

Table M-3 Multivariable association between liver markers and any incident cardiovascular disease events (subjects with NAFL). Values are hazard ratios (95%CI)

	Model 1	*p*	*Model 2*	*p*	*Model 3*	*p*
ALT, U/L	0.99 (0.97,1.02)	*0.446*	0.98 (0.968,1.01)	*0.236*	0.98 (0.96,1.01)	*0.219*
AST, U/L	1.01 (0.97,1.04)	*0.800*	1.00 (0.97,1.04)	*0.851*	1.00 (0.96,1.04)	*0.860*
GGT, log_2[a]	1.58 (1.15,2.17)	***0.005***	1.57 (1.13,2.18)	***0.007***	1.56 (1.08,2.28)	***0.019***
CK18, log_2[a]	0.91 (0.54,1.52)	*0.711*	0.91 (0.55,1.52)	*0.718*	0.98 (0.58,1.66)	*0.944*
APRI, log_2[a]	0.94 (0.52,1.70)	*0.840*	0.80 (0.43,1.51)	*0.495*	0.69 (0.36,1.31)	*0.691*
AST:ALT ratio	8.14 (0.77,85.48)	*0.081*	26.62 (2.03,349.29)	***0.012***	20.10 (1.36,296.91)	***0.029***
ELF score	1.10 (0.80,1.50)	*0.567*	1.19 (0.86,1.66)	*0.294*	1.20 (0.84,1. 07)	*0.317*
FIB4	1.06 (0.47,2.35)	*0.895*	0.92 (0.39,2.19)	*0.854*	0.70 (0.28,1.73)	*0.436*
NFS	0.96 (0.83,1.11)	*0.564*	0.98 (0.84,1.14)	*0.784*	0.97 (0.84,1.12)	*0.682*
Platelets, $x10^9$/L	1.00 (1.00,1.01)	*0.479*	1.00 (1.00,1.01)	*0.145*	1.01 (1.00,1.01)	*0.068*

ALT, AST, GGT, APRI, AST:ALT ratio, FIB4, NFS and platelets total n=319 incident CVD n=38; CK18 total n=295 incident CVD n=29; ELF total n=231 incident CVD n=26.

[a] APRI, CK18 and GGT analysed on the Log_2 scale for linearization, therefore odds ratios relate to a doubling of the marker.

Model 1 – Unadjusted; Model 2 - Adjusted for age and sex; Model 3 - Adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, body mass index, systolic blood pressure, diastolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and estimated glomerular filtration rate. Incident analysis additionally adjusted for prevalence cardiovascular disease at baseline.

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

Table M-4 Multivariable association between liver markers and incident coronary artery disease events (subjects with NAFL). Values are hazard ratios (95%CI)

	Model 1	*p*	*Model 2*	*p*	*Model 3*	*p*
ALT, U/L	0.98 (0.94,1.01)	0.188	0.97 (0.94,1.01)	0.127	0.96 (0.92,1.00)	0.065
AST, U/L	0.99 (0.94,1.04)	0.748	0.99 (0.94,1.04)	0.709	0.98 (0.93,1.03)	0.367
GGT, log_2[a]	1.47 (0.98,2.22)	0.063	1.46 (0.97,2.21)	0.072	1.36 (0.84,2.22)	0.212
CK18, log_2[a]	0.68 (0.35,1.32)	0.258	0.67 (0.35,1.28)	0.228	0.75 (0.38,1.50)	0.422
APRI, log_2[a]	0.58 (0.27,1.24)	0.159	0.56 (0.25,1.23)	0.145	0.43 (0.19,0.98)	0.044
AST:ALT ratio	19.53 (1.05,363.38)	**0.046**	38.00 (1.72,841.03)	**0.021**	28.96 (1.08,775.14)	**0.045**
ELF score	0.80(0.39,1.63)	0.538	0.80 (0.38,1.68)	0.553	0.76 (0.29,1.98)	0.574
FIB4	0.48 (0.14,1.63)	0.241	0.51 (0.14,1.81)	0.299	0.31 (0.08,1.22)	0.093
NFS	1.04 (0.86,1.25)	0.692	1.05 (0.87,1.27)	0.632	0.99 (0.81,1.20)	0.883
Platelets, $x10^9$/L	1.01(1.00,1.01)	0.076	1.01 (1.00,1.01)	0.060	1.01 (1.00,1.02)	0.015

ALT, AST, GGT, APRI, AST:ALT ratio, FIB4, NFS and platelets total n=319 incident CAD n=23; CK18 total n=295 incident CAD n=18; ELF total n=231 incident CAD n=15.

[a] APRI, CK18 and GGT analysed on the Log_2 scale for linearization, therefore odds ratios relate to a doubling of the marker.

Model 1 – Unadjusted; Model 2 - Adjusted for age and sex; Model 3 - Adjusted for age, sex, duration of diabetes, treatment of diabetes, lipid lowering drugs, blood pressure lowering drugs, deprivation (Scottish Index of Multiple Deprivation quintile), smoking status, excess alcohol consumption, body mass index, systolic blood pressure, diastolic blood pressure, HbA1c, HDL cholesterol, total cholesterol and estimated glomerular filtration rate. Incident analysis additionally adjusted for prevalence cardiovascular disease at baseline.

ALT alanine aminotransferase; **APRI** aspartate aminotransferase to platelet ratio index; **AST** aspartate aminotransferase; **CK18** cytokeratin-18; **ELF** Enhanced Liver Fibrosis; **FIB4** Fibrosis-4 score; **GGT** gammaglutamyl transferase; **NFS** NAFLD Fibrosis Score

Appendix N Mortality in the ET2DS vs general population

Table N-1 Numbers of deaths in the age group 60-74 years

	Scotland	*Lothian*	*ET2DS*
2008	14,060	1,794	26*
2009	13,623	1,724	22[#]
2010	13,563	1,808	29[$]
Total	41,246	5,326	51

Table N-2 Population at risk in the age group 60-74 years

	Scotland	*Lothian*	*ET2DS*
2008	781,273	107,516	1,063*
2009	796,971	109,939	1,037[#]
2010	809,394	109,939	1,015[$]
Total	2,387,638	327,394	2,052

Table N-3 Death rate per 1000 population/year in the age group 60-74 years

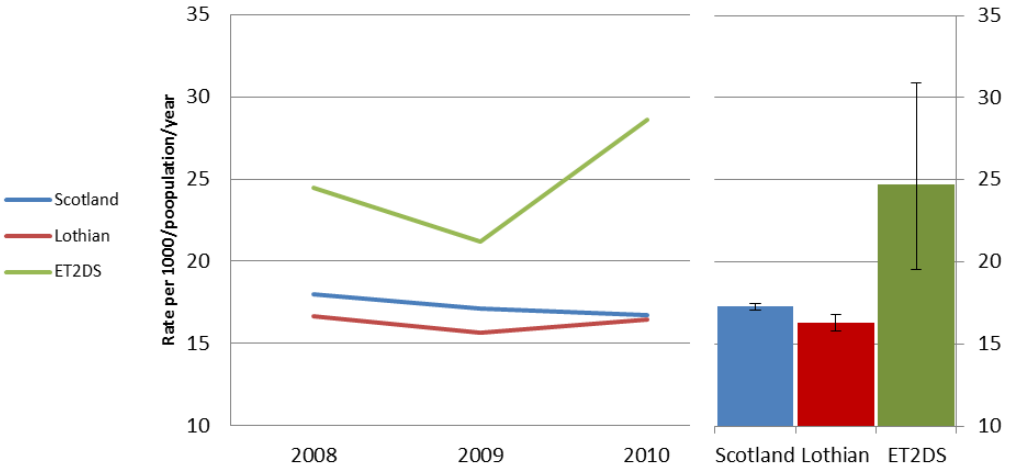
	Scotland	*Lothian*	*ET2DS*
2008	18.0	16.7	24.5*
2009	17.1	15.7	21.2[#]
2010	16.8	16.4	28.6[$]
Total	17.3	16.3	24.7

***** In 2008 the ET2DS population were aged 62-76 years

[#] In 2009 the ET2DS population were aged 63-77 years

[$] In 2010 the ET2DS population were aged 64-78 years

Figure N-1 Death rates in Scotland, Lothian and the ET2DS in people aged 60-74 years, 2008-2010. (A) Rates over time, (B) Overall rates 2008-10 with 95% CI



Appendix O Published papers related to this thesis

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ORIGINAL ARTICLE

Non-invasive hepatic biomarkers (ELF and CK18) in people with type 2 diabetes: the Edinburgh type 2 diabetes study

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Keywords
cytokeratin-18 – enhanced liver fibrosis score – fatty liver disease – non-invasive hepatic biomarkers – type 2 diabetes mellitus

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Abstract

Background & Aims: Type 2 diabetes is an established risk factor for the presence and progression of fatty liver. Little is known about the distributions and correlates of hepatic non-invasive biomarkers in community-based populations with diabetes, unselected for liver disease. We aimed to identify the distribution of, and metabolic risk factors associated with serum cytokeratin-18 (CK18) and the Enhanced Liver Fibrosis score (ELF), in a large, representative cohort of people with type 2 diabetes (the Edinburgh Type 2 Diabetes Study, ET2DS). *Methods:* Nine hundred and thirty-nine ET2DS participants, aged 60–74 years underwent physical examination including ultrasound for assessment of liver fat. Representative subgroups were assessed for markers of chronic liver disease (CK18 and ELF). *Results:* CK18 values ranged from 29–993 U/L (median 102, IQR 76–137 U/L) and ELF scores ranged from 6.9–11.6 (mean 8.9, SD 0.8). Statistically significant associations were found between both biomarkers and a number of metabolic risk factors. Neither CK18 nor ELF was consistently or strongly associated with established hepatic risk factors (alcohol excess, hepatotoxic medication use and positive immunology titres). *Conclusions:* We identified the distribution of CK18 and ELF in a large cohort of older people with type 2 diabetes and showed that these markers are associated with an adverse metabolic risk factor profile, although much of the variation in biomarkers remained unexplained. Prospective studies are required to determine the extent to which CK18 and/or ELF predict the development of symptomatic liver disease and to identify additional risk factors which may influence the development of advanced liver disease in people with type 2 diabetes.

Type 2 diabetes is an established risk factor for fatty liver in Western countries (1). The commonest cause of fatty liver, non-alcoholic fatty liver disease (NAFLD), ranges from simple steatosis (non-alcoholic fatty liver, NAFL), through to liver inflammation (non-alcoholic steatohepatitis, NASH) and on to NASH with liver scarring (fibrosis) and cirrhosis (end stage fibrosis). The consequences of the more advanced stages of NAFLD, including cirrhosis, liver failure and hepatocellular carcinoma (2) are becoming increasingly common; NAFLD is the third most frequent indication for liver transplantation in the USA and transplants performed with NAFLD as the primary aetiology rose from 1.0% in 2001 to 8.5% in 2009 (3). Even without transplant, NASH and advanced stages of liver disease are associated with higher individual healthcare costs (4). In type 2 diabetes, significant mortality related to chronic liver disease (CLD) has been reported (5) with a standardised mortality ratio (SMR) of 2.52 for cirrhosis compared with the general population in a European cohort (compared with a SMR for cardiovascular disease of 1.34) (6).

Given the potential burden caused by symptomatic (advanced) liver disease in the diabetic general population, there is a need to investigate potential methods of identifying asymptomatic stages of the condition in adults with diabetes, as indeed is the case for other high risk sub-groups within the general population. Liver biopsy, which to date has been the mainstay of liver fibrosis

diagnosis, has limited usefulness in the investigation of large groups of people from healthy populations (i.e. those unselected for liver disease) because of its invasive nature, complication rates (7), sampling errors (8) and interobserver variability (8). Therefore, interest is increasing in the use of non-invasive biomarkers of liver inflammation and fibrosis which might be useful in the identification of particularly high risk groups of individuals.

In this study, we sought to determine the distribution, and factors influencing levels of, two promising non-invasive liver markers, serum cytokeratin-18 (CK18) and the Enhanced Liver Fibrosis (ELF) score. These markers have previously been validated for hepatic inflammation and fibrosis, respectively, against liver biopsy in patients with established chronic liver disease attending tertiary care settings. CK18, a caspase cleaved fragment released by injured hepatocytes and a measure of hepatic cell damage such as inflammation in NASH, is raised in patients with CLD compared with people without CLD (9) and can differentiate between steatosis and NASH (10–12) in patients with NAFLD (13–15). ELF uses an extra-cellular matrix panel (hyaluronic acid (HA), N-terminal pro-peptide of collagen type III (P3NP) and tissue inhibitor of metalloproteinase-1 (TIMP-1) to quantify fibrosis and has been validated for use in patients with NAFLD (16) with increasing accuracy for severe fibrosis detection compared to earlier stages. However, neither biomarker has been validated in general population-based cohorts, nor in diabetic populations, an important issue which has been hampered to date by lack of information on their distribution and clinical correlates in such populations.

A recent study in South Korea reported on the normal distribution of ELF in adults without known CLD (13). This unique study of a large group of patients with type 2 diabetes, unselected for liver disease, investigates the distribution and clinical correlates of non-invasive markers of liver fibrosis and inflammation. This adds to previous studies which have focussed on hospital out patient settings and the use of liver biopsy (14, 15). Such information is necessary for the purpose of screening and treatment of undiagnosed liver disease in diabetic patients and also to underpin further research into the causes and consequences of asymptomatic liver disease in adults with diabetes.

Patients and methods

The Edinburgh type 2 diabetes study

Recruitment and examination of ET2DS subjects have been published elsewhere (17). In brief, 1066 men and women aged 60–75 years were recruited at random from the Lothian Diabetes Register. ET2DS participants have been shown previously to be representative of all those randomly selected to participate in this study ($n = 5454$), and of the target population of older people with type 2 diabetes living in the general population (18). A year after recruitment and baseline examination, 939 participants (88%) returned for further clinical and liver assessment (19, 20). Subjects returning at year 1 were similar to the full ET2DS population in terms of a number of variables including demographics, body fat measures, glucose and HbA1c measures, lipid profiles, blood pressure and medication use (18, 19).

Clinical examination

Clinical examination included a fasting blood sample for measurement of plasma glucose, HbA1c, total cholesterol, triglycerides, estimated glomerular filtration rate (eGFR), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase and platelets; measurement of height, weight, and waist circumferences; a self-administered questionnaire including questions on year of diabetes diagnosis, current medications, alcohol consumption, history of joint and liver disease. In addition, patients underwent abdominal ultrasound scan (USS) and those participants with evidence of hepatic steatosis or plasma liver enzymes above the laboratory reference limits received a 'liver screen' including: hepatitis B virus serology, hepatitis C virus serology, alfa-foetoprotein, ferritin and autoantibodies [antinuclear antibody (ANA), antimitochondrial antibody (AMA) and antismooth muscle antibody (ASmA)] (19). CK18 and ELF were measured in stored serum samples (−80°C), taken at the time of the liver USS.

Average alcohol intake per week over the previous year and history of alcohol excess were determined from two questions in the self-completion questionnaire, adapted from the AUDIT-C screening tool (21): 'How often did you have a drink containing alcohol in the past year?' (a drink was considered to be one and a half alcohol units); and 'How many drinks did you have on a typical day when you were drinking in the last year?'. Self-reported data on potential hepatotoxic medication use within the previous 6 months were confirmed by review of medical records.

Identifying prediagnosed liver disease

The presence of liver disease diagnosed prior to attendance at the research clinic was identified from data linkage to SMR01 general and acute inpatient discharge records (at NHS National Services Scotland, Information Services Division) and from questions on prior health condition in the patient questionnaires. Diagnoses were verified by review of medical records. Patients with confirmed chronic viral hepatitis, haemochromatosis and primary biliary cirrhosis were excluded from the final analyses as biomarkers are known to perform differently in these conditions.

Defining hepatic steatosis

Hepatic steatosis was determined by abdominal USS as described previously (22). The same sonographer,

Liver International (2013)

blinded to the participants' clinical history, undertook all scanning and grading. The liver was graded for markers of hepatic steatosis using established criteria: bright hepatic echo pattern (when compared to the right kidney), increased attenuation of the echo beam (visualised as poor imaging of the diaphragm or intrahepatic vessels) and the presence of focal fatty sparing (19, 23–25). In a subset, sonographic steatosis was validated using magnetic resonance spectroscopy with an optimal fat fraction cut-off of 6% (22) following which grades were defined as: normal (fat fraction <6.1%) or significant steatosis (fat fraction >6%). Radiological signs of cirrhosis were also noted and spleen size was measured in cm.

Laboratory measurements

CK18 was measured using the M30-Apoptosense® ELISA (PEVIVA AB, Stockholm, Sweden) at the Biomedical Research Unit laboratory (University of Nottingham, UK) and ELF using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics Inc, New York, USA) at the iQur laboratory (London, UK). The individual ELF markers are combined using the algorithm ELF=2.588 + (ln(HA)*0.681) + (ln(P3NP)*0.775) + (ln(TIMP1)*0.494) (16). All other biochemical variables were analysed using a Vitros Fusion chemistry system (Ortho Clinical Diagnostics, Bucks, UK) at the Western General Hospital (Edinburgh, UK). AST/ALT ratio was calculated as AST(U/L)/ALT(U/L) and the aspartate to platelet ratio index (APRI) was calculated as [[AST(U/L)/upper limit normal]/platelets($x10^9$/L)]*100.

Data analysis

Data were analysed using IBM SPSS Statistics for Windows, Version 19.0. (IBM Corp., Armonk, NY, USA). Alcohol excess was defined according to established criteria as alcohol intake >14 units/week (female) or >21 units/week (male) (26), or participant self-report of current/previous alcohol excess (2). Use of hepatotoxic medication included the use of (non-topical) glucocorticoids for >2 weeks, isoniazid, methotrexate, amiodarone, or tamoxifen within the 6 months prior to USS (2, 27). Clinically significant positive immunology titres were defined as ASmA titre >1:160 or AMA titre >1:40 (2, 28). CKD was defined as eGFR<60 mls/min/1.73 m^2. Patients were considered to have arthritis if they reported a history of osteoarthritis, rheumatoid arthritis, scleroderma or any other joint disease. Continuous variables were assessed for normality with CK18, duration of diabetes and triglycerides requiring transformation for analysis.

The associations of CK18 and ELF with the following were examined, (i) duration of diabetes and diabetes treatment categorised as diet-controlled, oral antihypoglycaemic agents (OAHA) only or insulin ± OAHA,), (ii) metabolic variables (total cholesterol, triglycerides, fasting glucose, HbA1c, BMI calculated as weight (kg)/height $(m)^2$ and waist circumference), (iii) steatosis on USS and, (iv) established risk factors for CLD (alcohol excess, hepatotoxic medication, positive immunology). The influence of CKD and arthritis (on circulating biomarker level) was assessed by analysing their prevalence in the highest and lowest quintiles of each biomarker. Analyses were undertaken on (i) all subjects, (ii) subjects with steatosis (defined as the presence of steatosis on USS) and (iii) subjects with NAFL (defined as the presence of steatosis on ultrasound without alcohol excess, use of hepatotoxic medication or raised autoantibodies).

Univariate analysis of potential risk factors was undertaken using Pearson's correlation and ANOVA adjusting for age and sex. Multivariate analysis was undertaken using linear regression both unadjusted and fully adjusted for age, sex and established hepatic risk factors.

A sensitivity analysis addressing missing CK18 and ELF data was performed using multiple imputation by chained equations (29). Data were considered to be missing completely at random as they were missing because of technical problems or insufficient stored sample.

Ethical approval was obtained from the Lothian Research Ethics Committee and all subjects gave written informed consent.

Results

Subject characteristics

Of the 939 ET2DS participants who underwent liver ultrasound and further physical and liver assessment, 899 did not have prediagnosed liver disease or CLD on screening and were considered for inclusion in the current analysis (15 subjects were excluded because of prediagnosed CLD, a further 3 because of CLD on screening and 22 because they did not have a liver screen when indicated). Eight hundred and twenty-five and 568 of these subjects underwent measurement of CK18 and ELF, respectively, and form the primary study populations for this paper. A number of ELF measurements were missing because of inadequate sample volumes. Details of the flow of patients through the study are shown in Fig. 1 and characteristics of the study populations are described in Table 1. Compared with all subjects undergoing ultrasound examination, the populations with CK18 and ELF data available had similar clinical and metabolic characteristics. Prevalence of steatosis was 56.8% and the prevalence of NAFL (defined as the presence of steatosis on ultrasound without alcohol excess, use of hepatotoxic medication or raised autoantibodies) was 31.5%.

Biomarker distributions

CK18 values ranged from 29 to 993 U/L (median 102, IQR 76–137 U/L) and ELF scores ranged from 6.9 to

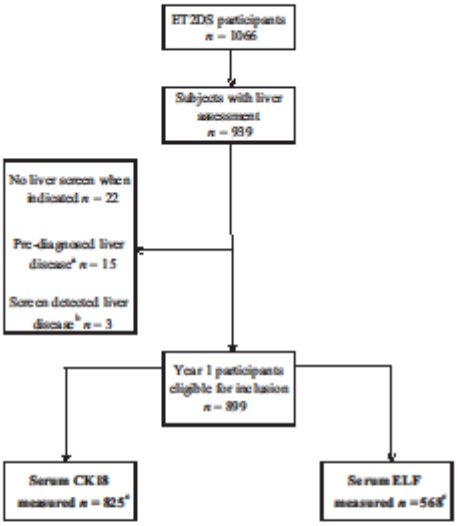

Fig. 1. Patient flow diagram.
[a]Pre-diagnosed liver diseases (n=15) included alcohol related liver disease (n=7), autoimmune hepatitis (n=2), primary biliary cirrhosis (n=2), haemochromatosis (n=1), granulomatous hepatitis (n=1), chronic cholangitis (n=1) and carcinoid tumour (n=1).
[b]Screen-detected liver disease included hepatitis B virus n=1, hepatitis C virus n=1, hepatocellular carcinoma n=1.
[c]Missing data values due to inadequate sample volumes.

11.6 (mean 8.9, SD 0.8). Distributions of CK18 were similar in men and women (medians 104 vs 100 respectively, $P > 0.05$) with ELF scores slightly lower in men (means 8.8, SD 0.7 and 9.0, SD 0.8, respectively, $P = 0.016$). ELF, but not CK18, increased significantly with age ($r = 0.28$, $P < 0.001$ and $r = -0.08$, $P > 0.05$ respectively) and the two markers significantly, though relatively weakly, correlated with each other ($r = 0.13$, $P = 0.002$).

Association of CK18 and ELF with hepatic steatosis and established hepatic risk factors

Subjects with hepatic steatosis ($n = 460$) had higher CK18 values compared with those without steatosis ($n = 365$) (medians 120.2 vs 87.7 U/L respectively, $P < 0.001$). The opposite was found for patients with NAFL (medians 87.4 vs 112.5 U/L, $P < 0.001$). ELF scores were similar in participants with steatosis ($n = 319$) compared with those without steatosis ($n = 259$) (means 8.88 vs 8.90, $P > 0.05$), with similar results for the presence of NAFL (means 8.95 vs 8.87, $P > 0.05$).

The associations of CK18 with established hepatic risk factors (excess alcohol intake, positive immunology titres and hepatotoxic medication use) are shown in Table 2, for all subjects ($n = 825$) and for those with steatosis ($n = 460$). After adjustment for age and sex, mean CK18 was significantly higher in subjects reporting excess alcohol intake but differences in positive immunology titres and hepatotoxic medication use were not statistically significant.

Similar findings for ELF are presented in Table 3, again for all subjects ($n = 568$) and for those with steatosis ($n = 319$). ELF appeared, if anything, slightly lower in subjects with established risk factors for liver dysfunction, but differences were not statistically significant.

Association of CK-18 and ELF with metabolic risk factors

Age- and sex-adjusted associations of CK18 and ELF with metabolic variables are shown in Tables 2 and 3. Higher CK18 levels were significantly associated with hyperglycaemia, increased body fat (higher BMI and waist circumference) and with higher serum triglyceride levels. Only the association with waist circumference remained statistically significant when analyses were restricted to subjects with NAFL.

Higher ELF levels were significantly associated with increasing duration of diabetes, with hyperglycaemia and with increased body fat. Compared with subjects who were treated with diet alone, mean ELF was significantly higher in subjects using OAHA alone (means 8.9 vs 8.7, $P = 0.012$) and in those using insulin (mean 9.2 vs 8.7, $P < 0.001$). The associations with ELF changed little when analyses were restricted to only subjects with steatosis or NAFL.

Characteristics of subjects in highest CK-18 and ELF quintiles

Since particularly high levels of CK18 and ELF may be diagnostic of clinically important liver inflammation and fibrosis respectively, we determined the clinical characteristics of subjects in the top biomarker quintiles (Table 4).

Compared with subjects in the bottom four quintiles, subjects in the highest CK18 quintile had significantly higher indices of hyperglycaemia, higher triglyceride levels and increased body fat. In addition, more were on intensive diabetes treatment (including insulin) and more reported drinking excess alcohol. When analyses were restricted to subjects with NAFL, no statistically significant differences were found (data not shown).

Subjects in the highest ELF quintile were slightly older, had longer diabetes duration and were more likely to require insulin therapy. These statistically significant differences persisted when the analyses were restricted to subjects with NAFL (age: mean 69.4 vs 67.8 years, $P = 0.010$; duration of diabetes: 6.97 vs 6.74 years, $P = 0.010$, on insulin therapy: 27.5 vs 13.5%, $P = 0.021$).

Subjects were also assessed for surrogate markers of advanced fibrosis. Subjects in the top ELF quintile had

Table 1. Characteristics* of all ET2DS participants undergoing liver assessment ($n = 939$) and groups with CK18 ($n = 825$) and ELF ($n = 568$) measurements. Values are mean (SD)/median (IQR) or proportion (n)

	All participants $n = 939$	CK18 participants $n = 825$	ELF participants $n = 568$
Age, years	68.9 (4.2)	68.8 (4.2)	68.7 (4.2)
Sex, % male	52.0 (488)	53.6 (442)	49.8 (283)
Duration of diabetes, years	7.0 (4.0–12.0)	7.0 (4.0–12.0)	7.0 (4.0–11.0)
HbA1c, %	7.19 (1.1)	7.19 (1.0)	7.20 (1.0)
HbA1c, mmol/mol	55.1 (11.7)	55.0 (11.4)	55.1 (12.3)
Fasting glucose, mmol/L	6.87 (2.3)	6.92 (2.3)	6.90 (2.3)
Diet controlled, % yes	19.4 (182)	19.8 (163)	18.8 (107)
OAHA use, % yes	64.9 (609)	65.3 (539)	66.7 (379)
Insulin therapy, % yes	15.8 (148)	14.9 (123)	14.4 (82)
BMI, kg/m^2	31.3 (5.7)	31.2 (5.6)	31.2 (5.7)
Waist circumference, cm	106.7 (12.8)	106.7 (12.7)	106.3 (12.5)
Serum total cholesterol, mmol/L	4.14 (0.8)	4.15 (0.8)	4.15 (0.8)
Systolic BP, mmHg	138.1 (18.5)	138.1 (18.2)	138.5 (18.1)
Diastolic BP, mmHg	74.1 (9.6)	74.4 (9.1)	74.6 (8.9)
Hepatic steatosis, % yes	56.8 (533)	55.8 (460)	56.2 (319)
NAFLD†, % yes	31.5 (296)	32.4 (267)	31.5 (179)
Alcohol, units/week	1..3 (0–10.1)	2.3 (0–10.1)	0.6 (0–10.1)
Alcohol excess‡, % yes	12.2 (114)	11.8 (97)	12.4 (70)
Current smoker, % yes	13.0 (122)	13.1 (108)	12.7 (107)

*All variables were measured concurrently at year 1 examination of the ET2DS, except for BMI and waist circumference which were measured at baseline.

†Defined as the presence of steatosis on ultrasound without alcohol excess, use of hepatotoxic medication or raised autoantibodies.

‡Defined as females >14 units/week, males >21 units/week or patient disclosed history of a current or prior alcohol problem.

HbA1c, glycosylated haemoglobin; OAHA, oral antihyperglycaemic agent.

significantly higher mean spleen size (10.6 vs 10.1 cm, $P = 0.020$), APRI (0.34 vs 0.26, $P < 0.001$), AST/ALT ratio (1.02 vs 0.93, $P = 0.011$) and lower platelet count (246 vs 267 $\times$ 10^9/L, $P = 0.005$).

Liver biomarkers were not significantly associated with other conditions known to potentially affect their levels in the circulation. The top and lower CK18 quintiles had similar proportions of subjects with CKD (19.4% vs 18.3%, $P = 0.74$) and the top and lower ELF quintiles had similar proportions of individuals with a diagnosis of arthritis (41.6% vs 38.0%, $P > 0.05$).

Multivariate analysis

In multivariate models adjusting for age, sex and established hepatic risk factors (Table 5), statistically significant positive predictors of CK18 were presence of hepatic steatosis, serum triglycerides and measures of increased body fat, hyperglycaemia and more intensive diabetes treatment. Similar predictors for ELF were duration of diabetes, more intensive diabetes treatment and body fat. In these models, r^2 ranged from 2.6 to 10.1% for risk factors influencing CK18 and from 12.7 to 13.7% for risk factors influencing ELF.

Because of the relatively large number of subjects in whom ELF (and to a lesser extent, CK18) data were unavailable (i.e. 'missing' at random), a sensitivity analysis was undertaken using multiple imputation (imputation data set included all 899 subjects eligible for inclusion in the analyses – see Fig. 1). The results confirmed those in the original data set with only minimal differences found in effect sizes and significance levels (data available on request).

Discussion

The strength of this study is the comprehensive assessment of CK18 and ELF in a population-based cohort of all older people with type 2 diabetes, and not just subjects selected primarily on the diagnosis of steatosis of the liver using recruitment from diabetes clinics at tertiary referral centres as in previous studies (14,30–32). We have provided diabetes-specific information on the distribution of these biomarkers, which is essential to inform further research on the clinical relevance of possible subclinical liver dysfunction in this high risk group. We also demonstrated that higher CK18 levels were associated with hepatic steatosis, excess alcohol intake, increased body fat, higher serum triglyceride and circulating glucose levels. ELF scores increased with age and duration of diabetes and were associated with increased body fat and more intensive diabetes treatment. A challenge in interpreting these results clinically is the lack of validated biomarker cut-points to diagnose hepatic inflammation and/or fibrosis in population-based cohorts. Despite this, our results suggest that at least a number of metabolic risk factors are likely to be associated with liver

Table 2. Association of CK18 with metabolic and established hepatic risk factors. Values are age- and sex-adjusted correlation coefficients or mean (SEM)

		All patients with CK18* available $n = 825$		Patients with steatosis $n = 460$		Patients with NAFLD $n = 378$	
Duration of diabetes*, years		0.015	NS	0.043	NS	0.037	NS
Treatment type							
Diet controlled		100.5 (1.8)		111.2 (3.0)		112.2 (3.4)	NS
OAHA use†		109.7 (1.1)	NS	127.4 (1.8)	NS	123.3 (1.8)	NS
Insulin therapy†		113.0 (2.4)	NS	136.8 (4.1)	0.026	126.2 (4.0)	NS
Metabolic risk factors							
Fasting glucose, mmol/L		0.093	0.008	0.112	0.016	0.062	NS
HbA1c, % and mmol/mol		0.064	NS	0.070	NS	0.048	NS
Body mass index, kg/m^2		0.117	<0.001	0.032	NS	0.085	NS
Waist circumference, cm		0.138	<0.001	0.061	NS	0.116	0.024
Serum total cholesterol, mmol/L		−0.051	NS	−0.041	NS	−0.073	NS
Serum triglycerides*, mmol/L		0.142	0.001	0.064	NS	0.100	NS
Established hepatic risk factors							
Excess alcohol intake‡	Yes	125.6 (3.0)		146.9 (4.7)			
	No	106.2 (1.0)	0.004	122.7 (1.5)	0.002		
Positive immunology§	Yes	120.5 (11.3)		97.5 (17.7)			
	No	110.2 (0.9)	NS	125.6 (1.4)	NS		
Hepatotoxic medication use¶	Yes	121.1 (5.1)		143.2 (7.6)			
	No	107.9 (0.9)	NS	124.7 (1.5)	NS		

Continuous variables analysed using Pearson's correlation, categorical variables analysed using univariate analysis of variance.
CK18, cytokeratin-18; HbA1c, glycosylated haemoglobin; OAHA, oral anthyperglycaemic agent; SEM, standard error of the mean.
*Analysed on the Log10 scale.
†vs diet controlled.
‡Defined as females >14 units/week, males >21 units/week or patient disclosed history of a current or prior alcohol problem.
§Defined as ASmA titre >1:160 or AMA titre >1:40.
¶Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic.

fibrosis and/or inflammation in people with type 2 diabetes.

While accepted as imperfect, especially in NAFLD (8), liver biopsy remains the gold standard for staging liver disease, but biopsy is not acceptable in large studies of asymptomatic participants. Cut-points for the non-invasive biomarkers used in this study have been well validated for staging liver disease in secondary care patient populations with an intrinsically higher prevalence of CLD, including NAFLD, but have not been validated as a diagnostic tool in either general population groups or in patients with type 2 diabetes. Given the considerable influence of disease prevalence on the predictive values of diagnostic tests, the results from hospital-based studies could not be transferred to our own community-based, 'low prevalence' population without resulting in an unacceptably high number of false positive and negative results. In terms of diabetic populations, it is not known whether ELF scores differ, on average, from non-diabetic populations, although there is also no known biological reason why this should be the case. For these reasons, we chose the top quintile of the biomarker distribution as our highest risk groups. Whilst the imprecision of such an approach in terms of diagnosing disease must be acknowledged, we have shown that the highest ELF quintile contained higher surrogate markers of advanced fibrosis, providing some confirmatory evidence that, at least for ELF, this group included a particularly high risk group of patients in terms of advanced liver disease. In addition, we were able to confirm that the presence of other conditions known to influence levels of the biomarkers do not appear to have a major effect on the results.

In terms of the distributions of potential inflammation and fibrosis biomarkers, our findings for CK18 were consistent with the assay literature (33). In developing the normal ranges for the serum CK18 assay, 200 'healthy' Swedish blood donors were tested; as in our study, the results showed similar levels in males and female with little change in levels with increasing age and an overall biomarker distribution similar to the one we found. In one study (33), a normal cut-point of the 80th percentile, or 145 U/L, was suggested, and this is also consistent with our finding (146 U/L). There is minimal literature examining ELF distributions in individuals unselected for liver disease. Yoo *et al.* suggest a normal range of 5.95–8.73 in South Korean subjects without known CLD (13). We found that ELF scores were very slightly lower in men and increased with age. In the absence of a biologically plausible reason to expect any difference in any of the components of ELF by sex, it is possible that the higher ELF scores in

Table 3. Association of ELF with markers of diabetes, the metabolic syndrome and liver dysfunction at year 1. Values are age- and sex-adjusted correlation coefficients or mean (SEM)

		All patients with ELF available $n = 568$		Patients with steatosis $n = 319$		Patients with NAFLD $n = 259$	
Duration of diabetes*, years		0.138	0.001	0.181	0.001	0.167	0.008
Treatment type							
Diet controlled		8.71 (0.07)		8.68 (0.10)		8.73 (0.11)	
OAHA use†		8.91 (0.04)	0.012	8.86 (0.05)	NS	8.89 (0.05)	NS
Insulin therapy†		9.15 (0.08)	<0.001	9.21 (0.10)	<0.001	9.23 (0.11)	0.002
Metabolic risk factors							
Fasting glucose, mmol/L		0.048	NS	0.025	NS	−0.006	NS
HbA1c, % and mmol/mol		0.090	0.033	0.048	NS	0.074	NS
Body mass index, kg/m^2		0.170	<0.001	0.237	<0.001	0.252	<0.001
Waist circumference, cm		0.148	<0.001	0.175	0.002	0.192	0.002
Serum total cholesterol, mmol/L		−0.035	NS	−0.089	NS	−0.068	NS
Serum triglycerides*, mmol/L		−0.008	NS	−0.116	NS	−0.140	NS
Established hepatic risk factors							
Excess alcohol intake‡	Yes	8.74 (0.09)		8.69 (0.11)			
	No	8.93 (0.03)	NS	8.91 (0.04)	NS		
Positive immunology§	Yes	8.61 (0.30)		8.31 (0.50)			
	No	8.90 (0.03)	NS	8.89 (0.04)	NS		
Hepatotoxic medication use¶	Yes	8.85 (0.17)		8.77 (0.20)			
	No	8.91 (0.03)	NS	8.89 (0.04)	NS		

Continuous variables analysed using Pearson's correlation, categorical variables analysed using univariate analysis of variance.
CK18, cytokeratin-18; HbA1c, glycosylated haemoglobin; OAHA, oral antihyperglycaemic agent; SEM, standard error of the mean.
*Analysed on the Log10 scale.
†vs diet controlled.
‡Defined as females >14 units/week, males >21 units/week or patient disclosed history of a current or prior alcohol problem.
§Defined as ASmA titre >1:160 or AMA titre >1:40.
¶Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic.

females may truly represent more advanced liver disease. The components of ELF (HA, TIMP-1 and P3NP) are all related to extra-cellular matrix turnover and are not exclusive to the liver. As a result, one might expect an increase in ELF with age, both because of the greater time in which liver fibrosis has had to develop (34) and because of the increasing prevalence of unrelated causes of raised analytes and indeed, consistent with our own findings, an early study examining HA and P3NP found higher levels in 'healthy' elderly people compared with younger participants (35).

Our finding of associations of hepatic steatosis, raised serum triglycerides and increased body fat, hyperglycaemia and more intensive diabetes treatment with CK18, and of duration of diabetes, more intensive diabetes treatment and body fat with ELF, support the possibility that poor diabetes control and a worse metabolic profile may be increasing the risk of developing CLD. Our findings contrast those of a recent liver biopsy study (14) in people with type 2 diabetes, in which high rates of both NASH (78%) and moderate fibrosis (34–60%) were detected, but which did not find associations between diabetes-related/metabolic factors and NASH or liver fibrosis. However, this biopsy study was small ($n = 98$) and focused on patients at the severe end of the diabetes spectrum attending a tertiary referral hospital.

The cross-sectional nature of our study limits any temporal inference; it is not possible to determine whether metabolic factors are a risk factor for liver disease or vice versa. However, if causal relationships were to be confirmed, this would have important implications for strategies aimed at CLD risk reduction e.g. losing/redistributing fat and reducing insulin resistance. Although associations between the biomarkers and metabolic factors appeared relatively weak, addressing even weak risk factors for disease could be beneficial at a population level, especially if those risk factors are highly prevalent.

In addition to the association of CK18 and ELF with metabolic risk factors, we were also interested in their association with steatosis and established hepatic risk factors. We found that subjects with hepatic steatosis had higher CK18 levels, but not ELF, compared with non-steatotic individuals. This is perhaps unsurprising given that CK18 levels rise with increasing hepatic inflammation as a by-product of hepatocellular apoptosis and that according to established models of NAFLD progression (27, 36), initial development of hepatic steatosis is followed by NASH and then hepatic scarring with steatosis typically receding as fibrosis progresses. Conversely, patients fulfilling the criteria for NAFL had significantly lower levels. This suggests that the

Table 4. Risk factors in highest vs lower quintiles of CK18 and ELF. Values are mean (SEM) or proportion (%)

	CK18 Quintile 1-4 $n = 660$ (<146.6 U/L)	CK18 Quintile 5 $n = 165$ (≥146.6 U/L)	P	ELF Quintile 1-4 $n = 455$ (score <9.5)	ELF Quintile 5 $n = 113$ (score ≥9.5)	P
Demographics						
Age	69.0 (0.16)	68.2 (4.6)	NS	68.3 (0.19)	70.6 (0.37)	<0.001
Sex, % male	54.5% (360)	49.7% (82)	NS	51.4% (234)	43.4% (49)	NS
Duration of diabetes*, years	7.04 (0.08)	7.29 (0.16)	NS	6.75 (0.09)	7.93 (0.26)	0.028
Treatment type						
Diet controlled	22.0% (145)	10.9% (18)	0.001	20.2% (92)	13.3% (15)	NS
OAHA use	72.6% (479)	83.6% (138)	0.004	75.8% (307)	79.6% (90)	NS
Insulin therapy	13.9% (92)	18.8% (31)	NS	12.3% (56)	23.0% (26)	0.007
Metabolic risk factors						
Fasting glucose, mmol/L	6.80 (0.09)	7.36 (0.19)	0.008	6.86 (0.10)	7.07 (0.25)	NS
HbA1c, %	7.14 (0.04)	7.36 (0.08)	0.013	7.19 (0.05)	7.22 (0.10)	NS
HbA1c, mmol/mol	54.5 (0.44)	57.0 (0.91)		55.1 (0.53)	55.4 (1.09)	
Body mass index, kg/m^2	31.0 (0.22)	32.3 (0.45)	0.009	31.0 (0.26)	31.8 (0.55)	NS
Waist circumference, cm	106.2 (0.50)	108.7 (0.97)	0.020	105.9 (0.58)	107.7 (1.22)	NS
Total cholesterol, mmol/L	4.15 (0.04)	4.13 (0.07)	NS	4.17 (0.04)	4.09 (0.09)	NS
Triglycerides*, mmol/L	1.42 (0.01)	1.68 (0.03)	0.001	1.44 (0.02)	1.52 (0.03)	NS
USS detected hepatic steatosis						
Steatosis	49.4% (326)	81.2% (134)	<0.001	56.9% (259)	53.1% (60)	NS
Established hepatic risk factors						
Excess alcohol intake†	10.3% (68)	17.6% (29)	0.014	13.4% (61)	8.0% (9)	NS
Positive immunology titres‡	0.7% (4)	1.2% (2)	NS	1.4% (6)	0% (0)	NS
Hepatotoxic medication use§	3.5% (23)	4.2% (7)	NS	3.5% (16)	2.7% (3)	NS

Continuous variables analysed using Students *t*-test, categorical variables analysed using Chi-square.

CKD, Chronic kidney disease; CK18, cytokeratin-18; ELF, European Liver Fibrosis panel; HbA1c, glycosylated haemoglobin; OAHA, oral antihyperglycaemic agent; SEM, standard error of the mean.

*Analysed on the Log10 scale.

†Defined as females >14 units/week, males >21 units/week or patient disclosed history of a current or prior alcohol problem.

‡Defined as ASmA titre >1:160 or AMA titre >1:40.

§Defined as the use of (non-topical) glucocorticoids for >1 week, isoniazid, methotrexate, amiodarone or tamoxifen within the 6 months prior to the year 1 clinic.

alternative causes of steatosis (hepatotoxic medications, alcohol and strongly positive autoantibody titres) are driving the inflammatory element, with those patients with NAFL having a more benign course. We found little evidence of a strong association between hepatic risk factors and either CK18 or ELF. Lack of associations may be explained, at least in part, by the small numbers of study participants with high levels of the hepatic risk factors and by lack of consensus around the precise level of risk factor which should be used to establish increased risk. We defined alcohol excess using cut-points which are consistent with the published literature in the UK, only six participants had positive autoantibodies and we were unable to find any consensus in the literature on how best to define hepatotoxic medications in terms of what types, duration and dosage are required to have a significant effect on the liver (2).

One of the most consistent findings in this study was the association of both biomarkers with measures of increasing body fat. Previous studies have shown a direct association between liver fat and hepatic inflammation, with the latter increasing proportionately according to liver fat volume (37). It has been proposed that this effect is mediated though the direct release of toxic free fatty acid by hepatic fat and through altered lipid partitioning within hepatocytes, mitochondrial dysregulation, generation of reactive oxygen species, lipid peroxidation and endoplasmic reticulum stress (38). Given the relationship between visceral fat and inflammation, our finding of increased body fat in patients in the highest CK18 quintile is consistent with proposed underlying mechanisms of hepatic inflammation (39, 40). In addition to increased body fat, subjects in the top CK18 quintile also had higher fasting glucose and plasma HbA1c levels, as well as more intensive diabetes treatment modalities. These factors may be considered as surrogates of beta cell failure and worsening insulin resistance, which is in turn related to hepatic inflammation through increased lipolysis, increased free fatty acid presence in the liver and ultimately oxidative stress (41). As in other studies (42, 43), we found higher triglyceride levels with increased CK18 levels, which

Table 5. Multivariate association of risk factors with CK18 and ELF. Values are standardised beta coefficients (95% CI)

CK18	Model 1	*P*	R^2	Model 2	*P*	R^2
Hepatic steatosis	0.302 (0.23 to 0.37))	<0.001	0.090	0.292 (0.22 to 0.36)	<0.001	0.101
Triglycerides*, mmol/L	0.148 (0.06 to 0.23)	<0.001	0.023	0.152 (0.07 to 0.23)	<0.001	0.050
Waist circumference, cm	0.142 (0.07 to 0.21)	<0.001	0.019	0.128 (0.06 to 0.20)	0.001	0.034
Fasting glucose, mmol/L	0.098 (0.03 to 0.17)	0.006	0.010	0.090 (0.02 to 0.16)	0.011	0.028
Body mass index, kg/m^2	0.100 (0.03 to 0.17)	0.006	0.010	0.098 (0.02 to 0.17)	0.009	0.027
Diet controlled	−0.085 (−0.16 to −0.02)	0.017	0.007	−0.091 (−0.16 to −0.02)	0.011	0.027
HbA1c,% or mmol/mol	0.086 (0.02 to 0.16)	0.034	0.007	0.081 (0.01 to 0.15)	0.025	0.026
Any OAHA use	0.079 (0.01 to 0.15)	0.029	0.006	0.085 (0.01 to 0.16)	0.018	0.026
Total cholesterol, mmol/L	−0.055 (−0.14 to 0.03)	NS	0.003	−0.062 (−0.15 to 0.02)	NS	0.028
Insulin therapy	0.030 (−0.04 to 0.10)	NS	0.001	0.028 (−0.05 to 0.10)	NS	0.019
Duration of diabetes*, years	0.010 (−0.06 to 0.08)	NS	–	0.017 (−0.06 to 0.09)	NS	0.019
ELF	**Model 1**	***P***	**R^2**	**Model 2**	***P***	**R^2**
Body mass index, kg/m^2	0.154 (0.07 to 0.24)	<0.001	0.024	0.187 (0.10 to 0.27)	<0.001	0.137
Duration of diabetes*, years	0.149 (0.06 to 0.23)	0.001	0.022	0.124 (0.04 to 0.21)	0.003	0.120
Insulin therapy	0.143 (0.06 to 0.23)	0.001	0.019	0.152 (0.07 to 0.24)	<0.001	0.127
Diet controlled	−0.110 (−0.20 to −0.02)	0.012	0.012	−0.116 (−0.20 to −0.03)	0.006	0.118
Waist circumference, cm	0.097 (0.01 to 0.19)	0.032	0.009	0.155 (0.07 to 0.24)	<0.001	0.126
Any OAHA use	0.059 (−0.03 to 0.15)	NS	0.003	0.064 (−0.20 to 0.15)	NS	0.109
HbA1c,% or mmol/mol	0.047 (−0.04 to 0.13)	NS	0.002	0.070 (−0.01 to 0.16)	NS	0.112
Hepatic steatosis	−0.029 (−0.12 to 0.06)	NS	0.001	0.014 (−0.07 to 0.10)	NS	0.105
Triglycerides*, mmol/L	−0.025 (−0.13 to 0.08)	NS	0.001	−0.001 (−0.10 to 0.10)	NS	0.088
Fasting glucose, mmol/L	0.018 (−0.07 to 0.11)	NS	–	0.030 (−0.05 to 0.11)	NS	0.107
Total cholesterol, mmol/L	−0.003 (−0.11 to 0.10)	NS	–	−0.042 (−0.15 to 0.06)	NS	0.091

*Analysed on the Log10 scale.
Model 1 - Unadjusted model, individual variables with no adjustment.
Model 2 - Individual variables adjusted for age, sex, alcohol excess, hepatotoxic medication use and strongly positive autoantibodies.
CK18, cytokeratin-18; ELF, European Liver Fibrosis panel; HbA1c, glycosylated haemoglobin; OAHA, oral antihyperglycaemic agent.

would be consistent with the theory of free fatty acids driving lipid accumulation in the form of triglycerides in the liver in NAFL.

In conclusion, we have provided important new information on the distribution of CK18 and ELF in an elderly diabetic population unselected for liver disease. Also, we have provided evidence that CK18 and ELF are increased in those people with type 2 diabetes who have a more adverse metabolic profile, including higher levels of body fat, while established risk factors for CLD were not found to have a major influence of levels of the biomarkers. These findings could help identify particularly high risk groups within the diabetic population who may benefit from increased surveillance in relation to development of CLD and/or from targeting of specific metabolic risk factors. Prospective studies are now required to determine the extent to which CK18 and/or ELF predict the development of symptomatic liver disease and to identify additional risk factors responsible for the development of advanced liver disease in people with type 2 diabetes.

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Research Article

JOURNAL OF HEPATOLOGY

Using non-invasive biomarkers to identify hepatic fibrosis in people with type 2 diabetes mellitus: The Edinburgh type 2 diabetes study

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Background & Aims: It is difficult to determine the different stages of non-alcoholic fatty liver disease without the use of invasive liver biopsy. In this study we investigated five non-invasive biomarkers used previously to detect hepatic fibrosis and determined the level of agreement between them in order to inform future research.

Methods: In the Edinburgh Type 2 Diabetes Study, a population-based cohort aged 60–74 years with type 2 diabetes, 831 participants underwent ultrasound assessment for fatty liver and had serum aspartate aminotransferase to alanine aminotransferase ratio (AST/ALT), aspartate to platelet ratio index (APRI), European Liver Fibrosis panel (ELF), Fibrosis-4 Score (FIB4) and liver stiffness measurement (LSM) measured.

Results: Literature based cut-offs yielded marked differences in the proportions of the cohort with probable liver fibrosis in the full cohort. Agreement between the top 5% of the distribution for each biomarker pair was poor. APRI and FIB4 had the best positive agreement at 76.4%, but agreement for all of the other serum biomarker pairs was between 18% and 34%. Agreement with LSM was poor (9–16%).

Conclusions: We found poor correlation between the five biomarkers of liver fibrosis studied. Using the top 5% of each biomarker resulted in good agreement on the absence of advanced liver disease but poor agreement on the presence of advanced disease. Further work is required to validate these markers against liver biopsy and to determine their predictive value for clinical liver-related endpoints, in a range of different low and high risk population groups.

Keywords: Hepatic fibrosis; Non-invasive biomarker tests; Non-alcoholic fatty liver disease; Type 2 diabetes mellitus.

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; CLD, chronic liver disease; NFS, NAFLD Fibrosis Score; BMI, body mass index; ET2DS, Edinburgh Type 2 Diabetes Study; LDR, Lothian Diabetes Register; USS, ultrasound scanning; TE, transient elastography; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HA, hyaluronic acid; PIINP, aminoterminal peptide of pro-collagen III; TIMP1, tissue inhibitor of matrix metalloproteinase 1; SCD, skin capsule distance; LSM, liver stiffness measure; CVH, chronic viral hepatitis; PBC, primary biliary cirrhosis; AST/ALT, ratio alanine aminotransferase to aspartate aminotransferase ratio; APRI, aspartate to platelet ratio index; ELF, European Liver Fibrosis panel; FIB4, Fibrosis-4 Score; kPa, kilopascals; NPV, negative predictive value; spec., specificity.

Introduction

Liver dysfunction in people with type 2 diabetes mellitus is thought to be mainly caused by non-alcoholic fatty liver disease (NAFLD). The earliest stage of NAFLD is simple steatosis but this can progress to non-alcoholic steatohepatitis (NASH) and ultimately to hepatic fibrosis, cirrhosis and the long term complications of chronic liver disease (CLD) such as hepatocellular carcinoma. The prevalence of NAFLD is thought to be higher in type 2 diabetes than in the general population [1–4]. Research focusing on the identification of fatty liver using ultrasound suggests a prevalence of around 34% in the general population [1]; in type 2 diabetes our own group found the prevalence to be 42.6% [2] and this figure may rise to 70% in more selected sub-populations of diabetes [3,4]. The prevalence of NASH and NASH-related fibrosis is much harder to determine as currently the only widely accepted diagnostic method is liver biopsy. However, it is difficult to justify performing liver biopsy to determine the severity of liver disease in community based subjects, including volunteers in research settings for two key reasons (i) there is considerable variability in sampling and histopathological interpretation due

to the small volume of tissue sampled (typically 0.002% of the liver) [5] and subjective semi-quantitative scoring systems [6–8], and (ii) biopsy is associated with an adverse outcome profile including pain, bleeding and rarely death [9–12]. Thus, there is considerable interest in the adoption of validated non-invasive markers of fibrosis into clinical practice. In the few biopsy studies of populations with type 2 diabetes, the prevalence of advanced fibrosis in those with NAFLD was 7–12% [13–15].

Non-invasive markers have been extensively validated in secondary care for the diagnosis of hepatic fibrosis either for specific underlying pathologies (e.g., the NAFLD Fibrosis Score, NFS) or with varying disease specific cut-offs. There are three broad groups of biomarkers: single markers, combination marker panels and imaging. Increasing numbers of scales and scores are being developed, with most studies reporting acceptable diagnostic accuracy (AUC >0.7) for individual methods in diagnosing the presence of hepatic fibrosis in NAFLD. However, their reliability and utility in identifying undiagnosed liver fibrosis in wider clinical practice and in research settings is yet to be determined given the limited studies in primary care [16,17]. Our group has previously shown [18] that the utility of many simple marker panels (BAAT score, BARD score, NFS) is limited in a population with type 2 diabetes by the inclusion of age, body mass index (BMI) and diabetes and led to over-estimation of the prevalence of fibrosis and high levels of indeterminate results.

In this study we investigated five biomarkers used previously to detect hepatic fibrosis in clinical populations with NAFLD. We aimed to determine the level of agreement between these biomarkers, in a large, representative, well-phenotyped population of people with type 2 diabetes mellitus (the Edinburgh Type 2 Diabetes Study, ET2DS).

Patients and methods

Study population

Full methods of the ET2DS have been published previously [19]. In brief, patients aged 60–74 years were selected at random from the Lothian Diabetes Register (LDR), a comprehensive register of patients with diabetes living in Lothian, Scotland. 1066 patients were recruited and attended a baseline clinic for physical examination. Study recruits have been shown previously to be largely representative 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 [20]. Participants who were able and willing (n = 939) attended a liver assessment 1 year after baseline, including liver ultrasound scanning (USS) [21]. Subjects who were still living were invited to a further detailed assessment approximately four years after recruitment; these subjects (n = 831) form the study population for the current analysis.

Ethical approval was obtained from the Lothian Research Ethics Committee and all subjects gave written informed consent.

Clinical examination and liver assessment

Clinical examination at the year 1 liver assessment and year 4 follow-up was similar to that performed in earlier phases of the study, described in detail previously [19]. In brief, patients underwent physical examination (including height and weight measurements); venepuncture; self-administered questionnaire (including alcohol consumption) and liver imaging (including USS and transient elastography (TE)). Plasma glucose, HbA1c, platelets and liver enzymes (including alanine aminotransferase (ALT), aspartate aminotransferase (AST)) and albumin were measured on a fasting blood sample using a Vitros Fusion chemistry system (Ortho Clinical Diagnostics, Bucks, UK). European Liver Fibrosis (ELF) panel – comprising hyaluronic acid (HA), aminoterminal peptide of pro-collagen III (P3NP) and tissue inhibitor of matrix metalloproteinase 1 (TIMP1) – was measured using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics Inc, New York, USA) on serum stored at −80 °C. USS was performed using a Sonoline Elegra Ultrasound Imaging System (Siemen's Medical Systems Inc, Washington, USA), software version 6, using a 3.5 MHz transducer. A phantom (411 LE 0.5, GAMMEX rmi Ltd, Nottingham, UK) as described and validated previously using magnetic resonance spectroscopy [21], hepatic steatosis was graded as present or absent based on standard criteria.

One dimensional TE was performed using a FibroScan (Echosens, Paris, France) machine at the year 4 follow-up visit only. A single operator was formally trained by Echosens personnel prior to commencement of the study. Initial ultrasound assessment allowed measurement of the skin-capsule distance (SCD). For SCDs <2.5 cm the M probe was used, for SCDs ≥ 2.5 cm the XL probe was used in accordance with recommended standard Fibroscan operating procedures. The TE probe was placed in an intercostal space overlying the liver with the patient in the supine position. Using ultrasound to guide positioning, an area of the liver that was at least 6 cm deep and free from large vessels was selected for investigation. The area measured was between 25 mm–65 mm below the surface of the skin for the M probe and 35 mm–75 mm for the XL probe. The operator aimed to obtain ten valid liver stiffness measurements (LSM) with a success rate of at least 60% and IQR <30% of the final (median) result. All scans were undertaken in the fasting state (minimum 4 h). Every six months the probes were serviced and calibrated.

Any patient with plasma liver enzymes above the upper reference limit, any abnormality on liver USS (including steatosis) or LSM >8 kPa underwent a liver screen including viral serology, alpha-feto protein, ferritin, autoantibodies, immunoglobulins, caeruloplasmin and α1-antitrypsin. In addition, pre-diagnosed liver disease was identified from NHS National Services Scotland, Information Services Division data linkage to SMR01 general and acute inpatient discharge records and from patient self-report questionnaires on prior health conditions. Any liver disease identified from linkage and the patient questionnaire was confirmed using individual patient medical records and patients with confirmed pre-diagnosed liver disease (chronic viral hepatitis, CVH, haemochromatosis and primary biliary cirrhosis, PBC) were excluded from the final analyses.

Data analysis

The five biomarkers/panels evaluated in this investigation were derived as follows:

- Aspartate aminotransferase to alanine aminotransferase ratio (AST/ALT) calculated as AST(IU/L)/ALT(IU/L).
- Aspartate to platelet ratio index (APRI) calculated as [AST(IU/L)/upper limit normal]/platelets($\times 10^9$/L)] × 100 [22].
- European Liver Fibrosis panel (ELF) calculated as 2.588 + (ln(HA)+ (ln(P3NP)×0.775) + (ln(TIMP1)×0.494) [23].
- Fibrosis-4 Score (FIB4) calculated as [age(years) × AST(IU/L)]/[platelets($\times 10^9$/L) × √ALT(IU/L)] [24].
- Liver stiffness measurement in kilopascals (kPa) expressed as the median TE value from at least ten valid measurements.

Absolute change in serum biomarker was defined as the change between the year 1 liver assessment and year 4 follow-up. Serum biomarker change was also defined categorically as: increased (increase of >5% of liver assessment value); decreased (decrease of >5% of liver assessment value); and stayed the same (absolute change within 5% of liver assessment value).

NAFLD was defined as the presence of hepatic steatosis on USS without alcohol excess or use of hepatotoxic medication and a negative liver screen. Alcohol excess was defined according to established criteria as alcohol intake >14 units/week (female) or >21 units/week (male) [25], or participant self-report of current/previous alcohol excess [26]. Use of hepatotoxic medication included the use of (non-topical) glucocorticoids for >2 weeks, isoniazid, methotrexate, amiodarone, or tamoxifen within the 6 months prior to USS [3,26]. Clinically significant positive immunology titres were defined as ASmA titre >1:160 or AMA titre >1:40 [26,27].

All patients with data available for APRI, AST/ALT ratio, ELF, and FIB4 were included in the analysis. All continuous variables were assessed for approximation to the normal distribution with APRI and FIB4 showing a skewed distribution. Correlation between biomarkers was analysed after standardisation to Z-scores, and adjusted for age and sex. Cronbach's alpha was used to examine the inter biomarker agreement (using standardised Z-scores). Student's *t* test or the Mann-Whitney U test were used to compare means and Chi-squared test to compare proportions.

Validated cut-offs to reliably exclude advanced fibrosis (≥metavir F3) in NAFLD were determined from the literature aiming to achieve negative predictive values (NPVs) 90–95%: APRI = 1.0, specificity (spec.) 89%, NPV 84% [22,28]; AST/ALT = 1.0, spec. 90%, NPV 89% [28]; ELF = 10.358, spec. 94%, NPV 90% [23]; FIB4 = 1.30, spec. 65%, NPV 95% [24,28]; and LSM = 8.7, spec. 83.2%, NPV 94.6% [29]. However, since these threshold levels cannot reliably be extrapolated from the predominantly secondary care settings in which they were validated to a general population setting, we also assessed agreement between biomarkers using the same highest percentile across all biomarker panels. Prior studies suggest that the prevalence of advanced fibrosis in type 2 diabetes patients attending outpatient clinics is at least 2–6% in all patients and 7–12% in those with NAFLD [13–15]. As a result we estimated that the underlying prevalence of significant hepatic fibrosis in the whole ET2DS cohort might be in the region of 5% and around 10% in those with NAFLD. We therefore compared the top 5% (and 10%) of scores for each biomarker for agreement in the entire cohort (and in those with NAFLD respectively). Individual 2 × 2 tables were calculated for the absence/presence of probable fibrosis (based on percentiles) for each pair of biomarkers. Due to the difficulties in interpreting markers of total agreement [30] (e.g., kappa statistics), we calculated positive agreement (agreement on the presence of fibrosis by both biomarkers) and negative agreement (agreement on the absence of fibrosis by both biomarkers) [31].

Correlation between absolute changes in serum biomarkers (i.e., between year 1 and year 4) was analysed after standardisation to Z-scores, and adjusted for age and sex. Categorical change in biomarker was analysed using the Chi-squared test to compare proportions.

Analysis was undertaken on both the whole population without pre-diagnosed liver disease and additionally in those with NAFLD (defined as the presence of hepatic steatosis on USS without alcohol excess, use of hepatotoxic medication or abnormal liver screen (Fig. 1)).

Results

Participants

831 participants (78% of the original cohort) attended the 4-year follow-up clinic. There were no significant differences between attenders at baseline and at the 4-year visit (Table 1). Of the subjects that did not attend the 4-year clinic (n = 235), 9 had withdrawn from the study, 14 were un-contactable, 124 were unable or unwilling to attend and 88 had died. Eleven patients were excluded from the analysis due to pre-diagnosed liver disease.

767 participants had a complete biomarker dataset (excluding LSM) available for analysis. Of these, valid LSM was reported for n = 650. Missing plasma and serum markers were missing at random. LSM was missing in 117 participants (15.3%) due to failure to obtain valid readings. Patients with missing LSM were significantly more obese, with poorer glucose control and more severe markers of liver dysfunction (Table 2). There were 282 (36.8%) participants fulfilling the criteria for NAFLD with a complete biomarker dataset, with LSM available on 248.

		Biomarker 1			
		Fibrosis present	Fibrosis absent	Total	
Biomarker 2	Fibrosis present	a	b	a + b	Positive agreement = 2a/(2a + b + c)
	Fibrosis absent	c	d	c + d	Negative agreement = 2d/(b + c + 2d)
	Total	a + c	b + d	a + b + c + d	

Fig. 1. Calculation of agreement statistics.

Biomarker distributions and agreement

Biomarker distributions for the full study population are available in the Supplementary data (Supplementary Fig. 1) and correlations between the biomarkers in Table 3 and Supplementary Fig. 2. After adjustment for age and sex, correlation was strong between APRI and FIB4 ($r = 0.92$) but all others were ≤0.5. The inter-item correlation for all five markers was just below acceptable ($\alpha = 0.67$) with minimal improvement with the removal of any marker. Correlations were similar in the NAFLD cohort.

Literature based cut-offs yielded marked differences in the proportions of the cohort with probable liver fibrosis in the full cohort: APRI 0.8%, AST/ALT ratio 22.4%, ELF 7.0%, FIB4 68.3%, and LSM 4.5%; and in the NAFLD subgroup: APRI 0.4%, AST/ALT ratio 16.7%, ELF 4.3%, FIB4 63.8%, and LSM 4.8%.

Agreement between the top 5% of the distribution for each biomarker pair was poor (Table 4). APRI and FIB4 had the best positive agreement at 76.4%, but agreement for all of the other biomarker pairs was between 9% and 32%. When the comparison groups were altered to reflect the top 3% and 7% of the biomarker distributions, then with the exception of APRI and FIB4, the agreement did not alter greatly (agreement 9–26% and 11–38% respectively). When analyses were restricted to the study population with NAFLD, agreement between the top 10% of the distribution for each biomarker pair remained generally poor (agreement for APRI and FIB4 was 68%, all other pairs between 14% and 36%), as did agreement for the top 5% and 15% of the distributions (agreements from 0% to 36% and from 21% to 36% respectively, with exception of APRI/FIB4).

The top 5% (10% in NAFLD) was suggestive of advanced liver dysfunction with clinical data implicating advanced fibrosis/cirrhosis with platelet counts being significantly lower and spleen size being significantly larger in the majority of cases (data not shown).

Negative agreement (agreement on the absence of fibrosis) was more consistent for both the full cohort analysis and the NAFLD subgroup (90–99%) with minimal change with the alternative cut-offs described. 2 × 2 tables for agreement using the top vigintiles are available in the Supplementary data (Supplementary Table 1).

Changes in biomarkers

Serum biomarker pairs from the year 1 and year 4 follow-up were available for 534 subjects (LSM was not undertaken at year 1). Six patients were excluded for pre-existing liver disease leaving an analysis cohort of 528 subjects. There were 183 (34.7%) participants fulfilling the criteria for NAFLD.

Following a mean follow-up of 3.5 years, mean percentage (sd) changes in serum biomarkers were: AST/ALT ratio −0.04 (0.25), APRI 0.19 (0.37), ELF 0.03 (0.08) and FIB4 0.18 (0.30). The changes in each biomarker (adjusted for age and sex) were

Table 1. Characteristics of full ET2DS study population and liver biomarker analysis cohort, at baseline, year 1, and year 4.

Variable	Full ET2DS cohort at baseline (n = 1066)	Analysis cohort (n = 767)	
		yr 1	yr 4
Age, yr	67.9 (4.2)	68.7 (4.1)	71.4 (4.2)
Sex, % male	51.3 (547)	52.8 (405)	52.8 (405)
Fasting glucose, mmol/L	7.56 (2.1)	6.89 (2.2)	7.75 (2.9)
HbA1c, %	7.40 (1.1)	7.19 (1.1)	7.34 (1.2)
HbA1c, mmol/mol	57.3 (12.2)	55.1 (11.6)	56.7 (13.1)
Duration of diabetes, yr	6.71 (3.9-11.3)	7.00 (4.0-12.0)	7.10 (7.6-14.9)
Diabetes treatment			
Diet alone, %	19.3 (193)	19.7 (144)	13.9 (106)
OAHA alone, %	63.4 (652)	65.5 (479)	65.3 (498)
Insulin therapy (± OAHA), %	17.3 (178)	14.8 (108)	20.8 (159)
BMI, kg/m^2	31.4 (5.7)	31.2 (5.6)*	31.3 (5.8)
Total cholesterol, mmol/L	4.31 (0.9)	4.16 (0.8)	4.25 (0.9)
HDL cholesterol, mmol/L	1.29 (0.4)	1.23 (0.3)	1.37 (0.4)
Systolic BP, mmHg	133.3 (16.4)	138.3 (18.3)	131.4 (17.9)
Diastolic BP, mmHg	69.1 (9.0)	74.6 (9.4)	69.1 (9.2)
Smoking, % never	38.2 (407)	39.1 (300)*	39.1 (300)
Platelets, x10^9/L	257.7 (68.9)	259.8 (67.7)*	229.8 (66.8)
Steatosis present, %	-	55.1 (423)	50.1 (384)
ALT, IU/L	-	33.9 (12.8)	36.0 (11.8)
AST/ALT ratio	-	0.94 (0.3)	0.85 (0.2)
APRI	-	0.24 (0.2-0.3)	0.28 (0.2-0.4)
ELF	-	8.89 (0.8)	9.12 (0.8)
FIB4	-	1.37 (1.1-1.8)	1.56 (1.2-2.0)
LSM, kPa	-	-	5.11 (2.6)

*Measured at baseline visit.
Values are mean (sd), median (IQR) or% (n).
ALT, alanine aminotransferase; APRI, aspartate aminotransferase-platelet ratio index; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; ELF, European Liver Fibrosis panel; FIB4, Fibrosis-4 Score; HbA1c, glycosylated haemoglobin; LSM, liver stiffness measurement; OAHA, oral anti-hyperglycaemic agent.

poorly correlated (AST/ALT:APRI $r = 0.23$, $p < 0.001$; AST/ALT:ELF, $r = 0.15$, $p < 0.001$; AST/ALT:FIB4 $r = 0.55$, $p < 0.001$; APRI:ELF $r = 0.12$, $p = 0.008$; ELF:FIB4 $r = 0.13$ $p = 0.003$) with the exception of APRI and FIB4 ($r = 0.88$, $p < 0.001$). The results were similar when limited to patients with NAFLD (Supplementary Fig. 3). When classified as increasing, staying the same and decreasing, agreement remained weak (35.0–54.7%), again with the exception of APRI and FIB4 (76.3% agreement).

Discussion

This study is the first population based study to compare the distribution of different non-invasive markers of liver fibrosis in patients with type 2 diabetes. Its strength lies in its population based approach allowing the results to be generalised to the wider type 2 diabetes population and not just patients at the more severe end of the diabetes spectrum and/or those with known fatty liver disease attending hospital clinics. We found poor correlation between the five biomarkers of liver fibrosis studied. Using the top vigintile (5%) of each biomarker resulted in excellent agreement on the absence of advanced liver disease but poor agreement on the presence of advanced liver disease.

An interesting finding of this cohort is the likely small number of patients with hepatic fibrosis. Based on strict application of validated biomarker cut offs used previously in non-diabetic populations to indicate the presence of fibrosis, the prevalence of significant fibrosis (Metavir F3+) appears to be between 1% and 68%, but is most likely less than 10%. Since NAFLD is increasingly reported as associated with type 2 diabetes, it is perhaps surprising (though also encouraging) to find that the prevalence of advanced liver disease may be low in this perceived high risk group. There are several possible reasons for a low prevalence of fibrosis in our study population. It may be related to specific cohort effects and survival bias, with only 'low risk' adults with type 2 diabetes surviving to the age of 60 years and thereby enabling participation in the study. It may be 'artefact', due to behaviour of the biomarkers in populations with diabetes, such that cut-points, which indicate fibrosis in a non-diabetic person, do not reflect the same level of fibrosis in a diabetic person. There is little literature on the distribution of biomarkers in exclusively diabetic populations, however, there are no clear biologically plausible reasons to expect different thresholds to operate. Thus, it may also reflect a truly low prevalence of fibrosis in people with diabetes. This has not been widely studied previously, but given the strong association between insulin insensitivity and hepatic fibrosis development [32] it may be that the intensive management of diabetic patients with insulin sensitising agents (metformin [33] and peroxisome proliferator-activated receptor-gamma agonists (thiazolidinediones) [34]) is attenuating

Table 2. Association of risk factors for missing liver stiffness measures.

	LSM missing n = 119	LSM present n = 648	*p* value
Age, yr	71.2 (4.3)	71.4 (4.1)	n.s.
Sex, male	37.0 (44)	55.7 (361)	<0.001
Body mass index, kg/m^2	35.2 (7.7)	30.8 (5.1)	<0.001
Skin capsule distance, cm	2.83 (0.9)	2.32 (0.5)	<0.001
Fibroscan probe, 'M'	42.0% (50)	65.6% (425)	<0.001
Glucose, mmol/L	8.21 (3.4)	7.66 (2.8)	n.s.
HbA1c, %	7.54 (1.3)	7.30 (1.2)	0.038
HbA1c, mmol/mol	59.0 (14.2)	56.3 (12.9)	
ALT, IU/L	36.6 (13.2)	35.8 (11.5)	n.s.
AST, IU/L	31.3 (12.2)	29.3 (9.9)	n.s.
GGT, IU/L	19.0 (12.0-48.5)	16.0 (9.5-27.0)	0.001
AST/ALT ratio	0.88 (0.3)	0.85 (0.2)	n.s.
APRI	0.30 (0.21-0.44)	0.28 (0.21-0.37)	n.s.
ELF	9.37 (1.0)	9.08 (0.8)	0.002
FIB4	1.65 (1.21-2.30)	1.55 (1.22-1.96)	n.s.

Values are mean (sd), median (IQR) or% (n).
ALT, alanine aminotransferase; APRI, aspartate aminotransferase-platelet ratio index; AST, aspartate aminotransferase; ELF, European Liver Fibrosis panel; FIB4, Fibrosis-4 Score; GGT, gamma-glutamyl transferase; HbA1c, glycosylated haemoglobin; n.s., not significant.

Table 3. Correlations between biomarkers.

A Full cohort (n = 767)

APRI	r = 0.30			
ELF	r = 0.24	r = 0.31		
FIB4	r = 0.53	r = 0.92	r = 0.30	
LSM[a]	r = 0.15	r = 0.20	r = 0.25	r = 0.16
	AST/ALT	**APRI**	**ELF**	**FIB4**

B NAFL cohort (n = 282)

APRI	r = 0.32			
ELF	r = 0.18	r = 0.32		
FIB4	r = 0.53	r = 0.93	r = 0.29	
LSM[b]	r = 0.15	r = 0.29	r = 0.26	r = 0.29
	AST/ALT	**APRI**	**ELF**	**FIB4**

[a] n = 648.
[b] n = 248.
All p <0.001.
Values are correlation coefficients, adjusted for age and sex.
APRI, aspartate aminotransferase-platelet ratio index; AST/ALT, aspartate aminotransferase-alanine aminotransferase ratio; ELF, European Liver Fibrosis panel; FIB4, Fibrosis-4 Score; LSM, liver stiffness measure.

the development of fibrosis. The probable low prevalence of fibrosis in this cohort is a limitation when analysing biomarker agreement and, due the well-established influence of disease prevalence on diagnostic test accuracy, it may be that there would be a higher biomarker agreement in populations with higher fibrosis prevalence [35].

In addition, 15% of LSM data was missing. The XL probe is believed to increase the success rates of obtaining ⩾10 valid TE readings from 56% to 75% [36] in obese populations. This is consistent with our 85% success in a mixed overweight and obese cohort. Despite the use of the XL probe, TE still appears to be limited by body habitus. Those patients with missing LSM had more severe diabetes profiles and higher alternative biomarkers of hepatic fibrosis. Hence, it was felt necessary to expand the majority of analysis to those patients with a full set of biomarkers excluding LSM in order to avoid biasing the sample.

Overall there was poor correlation between the biomarkers measured in this study. The only exception was APRI and FIB4 (r = 0.92 in full cohort and 0.93 in NAFLD subgroup, p <0.001), which is unsurprising given they have a number of components in common (AST level and platelets). Assessment of inter-item correlation (α = 0.67) determined that the five biomarkers were not consistent with each other in what they were measuring, with a score of α = 0.70 being the minimum score usually accepted. However, a minimum of 0.90 is often suggested for clinical practice [37]. This suggests some discrepancy in what the biomarkers are measuring. This seems plausible as AST/ALT, APRI and FIB4 are all similar in their composition, including markers of hepatocellular damage. Alternatively, ELF is measuring markers related to extracellular matrix turnover in fibrosis, and LSM is also examining structural properties of the liver through shear wave transmission.

A higher prevalence of fibrosis in the NAFLD cohort compared to the full cohort was expected. However, using validated cut-offs, our results showed the opposite. This probably reflects the natural history of NAFLD, in which fibrotic progression is often associated with steatosis regression and hence this group of advanced liver disease patients might not be captured by our definition of NAFLD. ELF scores and LSM values for the top vigintile were most in keeping with the values one would expect to find from previous published studies [13–15], however, with no reference standard (biopsy) it is impossible to establish, which of the

Table 4. Agreement between biomarker pairs for the top 5% or 10% of values, in the full cohort and NAFL subgroup.

A Positive agreement (presence of fibrosis) in the top 5% of the full cohort.

APRI	18.4%			
ELF	18.4%	31.6%		
FIB4	34.2%	76.3%	34.2%	
LSM	9.5%	12.7%	15.9%	12.7%
	AST/ALT	**APRI**	**ELF**	**FIB4**

B Negative agreement (absence of fibrosis) in the bottom 95% of the full cohort.

APRI	95.7%			
ELF	95.7%	96.4%		
FIB4	96.6%	98.8%	91.8%	
LSM	95.4%	95.5%	95.7%	95.5%
	AST/ALT	**APRI**	**ELF**	**FIB4**

C Positive agreement (presence of fibrosis) in the top 10% of the NAFL subgroup.

APRI	28.6%			
ELF	14.3%	28.6%		
FIB4	35.7%	67.9%	32.1%	
LSM	29.2%	20.8%	25.0%	25.0%
	AST/ALT	**APRI**	**ELF**	**FIB4**

D Negative agreement (absence of fibrosis) in the bottom 90% of the NAFL subgroup.

APRI	92.1%			
ELF	90.6%	92.1%		
FIB4	92.9%	96.5%	92.6%	
LSM	92.4%	91.5%	9.9%	91.9%
	AST/ALT	**APRI**	**ELF**	**FIB4**

APRI, aspartate aminotransferase-platelet ratio index; AST/ALT, aspartate aminotransferase-alanine aminotransferase ratio; ELF, European Liver Fibrosis panel; FIB4, Fibrosis-4 Score; LSM liver stiffness measure.

five biomarkers is the most accurate and to comment on the true prevalence of hepatic fibrosis using non-invasive biomarkers.

In addition, using validated cut-offs we found a wide range of fibrosis prevalence results for the different biomarkers. The reasons for the wide discrepancies are probably two-fold. Firstly, as demonstrated by the lack of inter-correlation, there is an inconsistency in what the different biomarkers/panels are measuring. Secondly, published fibrosis cut-offs from clinical validation studies do not appear to translate readily into research or clinical practice. The difficulty with this is that the predictive value of a test (unlike sensitivity and specificity) is influenced by the prevalence of underlying disease. Most validation cohorts have typically comprised a high proportion of patients with advanced liver disease selected from tertiary referral centres. Our study population has a presumed lower prevalence of advanced liver disease and consequently the predictive values are likely to be different. This resulting lower positive predictive value and higher negative predictive value would mean that literature based cut-offs are neither reliable nor directly comparable with one another in different patient cohorts such as ours. Without liver biopsy (the current reference standard) it is not possible to decide, which biomarker is "best suited" for diagnosing significant hepatic fibrosis in a lower prevalence population.

There are numerous other panel markers of hepatic fibrosis available, e.g., BARD, BAAT, and NFS. These are typically simple scoring systems using easily available plasma results and patient data. Previous work from our group has found that these scores are likely to overestimate the prevalence of hepatic fibrosis in populations similar to ours as they rely heavily on the incorporation of impaired glucose tolerance, age, and body mass index. For example, the prevalence of fibrosis using the BARD and BAAT scores was 92.6% and 79.3%, respectively, with the NFS predicting 16.4% fibrosis and 66.8% indeterminate [18]. It is therefore necessary to concentrate on the development of hepatic fibrosis markers that are independent of the underlying characteristics of the population under study.

As we observed for the cross-sectional distribution of serum biomarkers, changes in the biomarkers over 3.5 years were also poorly correlated with the exception of the similarly derived biomarkers (APRI and FIB4). Even after categorising change as increasing, staying the same or decreasing, agreement remained weak. This is not surprising given the weak associations in cross-sectional analysis, however this may also reflect the short follow-up period.

A further area which requires clarification is the most appropriate use of hepatic biomarkers in the general/healthy population. Without this information, it is hard to predict whether expected values are likely to differ in discrete populations, such as the elderly or people with diabetes. Normal routine liver function tests vary with age, sex and ethnicity [38,39], and therefore any fibrosis marker panel including these components might benefit from specific reference ranges that reflect the individual population.

At present, use of these non-invasive biomarkers is limited within low disease prevalence settings to excluding advanced liver disease. Further work is required to validate these markers for the presence of advanced liver disease against the liver biopsy, but more importantly to determine their predictive value for clinically relevant liver-related endpoints, such as hepatocellular carcinoma, oesophageal varices and cardiovascular outcomes, in a range of different low and high risk population groups, most notably community settings. The practical and ethical challenges of large scale liver biopsy in 'normal' patients persist, but emerging techniques, such as magnetic resonance elastography [40], have the potential to become a more acceptable reference standard.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

JRM designed the study, collected and analysed data, and wrote the manuscript. JAF researched data, contributed to the discussion, and reviewed/edited the manuscript. ING researched data and reviewed/edited the manuscript. LDN collected data and reviewed/edited the manuscript. SG designed the study and reviewed/edited the manuscript. RMW designed the study, collected data, and reviewed/edited the manuscript. CMR collected data and reviewed/edited the manuscript. MWJS designed the study, contributed to the discussion, and reviewed/edited the manuscript. JFP designed the study, contributed to the discussion, and reviewed/edited the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013.10.017.

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