Novel non-invasive markers, imaging and interventions for the diagnosis, characterisation and treatment of non-alcoholic fatty liver disease



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by

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Abstract

This DPhil thesis outlines research undertaken in non-alcoholic fatty liver disease (NAFLD).

Chapter 1 contains a literature review summarising current concepts in NAFLD including the burden of disease, clinical management including diagnosis and staging, and emerging therapeutic options to treat NAFLD. The first research chapter (Chapter 2) is an analysis of how patients with NAFLD are currently managed in clinical practice, the effectiveness of health interventions and of clinical outcomes measured by using currently available biomarkers. The outcomes and changes in liver and cardio-metabolic health markers of 165 patients with NAFLD who attended a secondary and tertiary metabolic hepatology (NAFLD) clinic at Oxford University NHS Foundation Trust over a median follow-up period of 13 months are presented. The analysis describes the burden of fatty liver disease in its various stages (steatosis, steatohepatitis (NASH), fibrosis, cirrhosis) across the Oxfordshire region. It outlines the use of non-invasive diagnostic and risk stratification tools including hepatic elastography, and how these change longitudinally at follow-up. Finally, as the quest for novel, specifically licenced therapies for NAFLD continues, this study outlines the interventions and approach used in the clinic including an analysis of changes to medical therapy including weight loss promoting agents such as glucagon-like peptide 1 (GLP-1) receptor agonists in patients with NAFLD.

The two subsequent research chapters explore two important themes arising from the clinic analysis. Chapter 3 outlines novel research which aims to help meet the need for improved diagnostic and staging tools for NAFLD, and in particular the need for novel non-invasive markers to allow for better risk stratification at diagnosis and for subsequent monitoring of the disease after therapy has been instigated. Drawing on previous work which implicates dysregulated glucocorticoid metabolism as a feature of NAFLD, with specific dysregulation varying across the spectrum of NAFLD, it investigates if the wider steroid metabolome can be used as a novel non-invasive biomarker tool to diagnose and stage NAFLD. This pilot

study is conducted in 275 individuals with biopsy proven disease across the spectrum of NAFLD, and explores both specific glucocorticoid metabolic pathways in these individuals as well as use of the 'global urinary steroid metabolome' coupled with machine learning techniques to determine NAFLD stage. The results show excellent promise and suggest that if validated, this technique could have future high utility in clinical practice.

Following on from Chapter 1 which revealed that around 20 % of patients with type 2 diabetes attending the NAFLD clinic were commenced on GLP-1 therapy, and that the GLP-1 agonist Liraglutide has been previously shown in research studies to be useful in patients with NASH, Chapter 4 investigates GLP-1 agonists as a novel therapy for NAFLD. What hitherto remains unclear is whether the benefit of GLP-1 therapy is solely due to its weight loss actions alone or whether there exist any direct GLP-1 effects on the liver. To seek to answer this question of any weight independent effects of Liraglutide in NASH, this chapter presents data from the 'Lifestyle and Liraglutide in NASH' (LiLi) clinical trial which contributes to the evidence base that may in due course inform medicines licensing agencies to licence GLP-1 agonist therapy as one the first treatments for NAFLD. 29 subjects with NASH were randomised to either Liraglutide or lifestyle intervention to induce weight loss and were followed for a minimum of 12 weeks. Both groups lost equal amounts of weight of approximately 5 % which allowed direct assessment of weight independent benefits. Data arising from metabolic tracer studies (deuterated water and ^{13}C-glucose to detail lipid and glucose handling within the liver), biochemical and body composition data are presented. Data on liver fat and inflammation measured using magnetic resonance spectroscopy and using novel multiparametric magnetic resonance imaging (LiverMultiScan™, Perspectum Diagnostics) pre and post weight loss intervention are also presented. This study finds that weight loss induced by Liraglutide is of equal efficacy and provides equivalent health benefits to losing weight via dietary lifestyle measures though this is only maintained whilst taking therapy. When considering that clinicians urgently require a range of tools at their disposal to tackle NAFLD and that weight loss and maintenance thereafter is difficult to

achieve, this study concludes that GLP-1 agonists could play an important role as a future licenced therapy for NAFLD.

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List of abbreviations

5αR1	Steroid 5α-reductase 1
5αR2	Steroid 5α-reductase 2
11β-HSD1	11β-hydroxysteroid dehydrogenase type one
11β-HSD2	11β-hydroxysteroid dehydrogenase type two
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under the curve
BMI	Body mass index
CO_2	Carbon dioxide
cT1	Corrected T1 (marker of liver inflammation and fibrosis)
CTIMP	Clinical trial of an investigational medicinal product
CVD	Cardiovascular disease
DNL	De novo lipogenesis
ELISA	Enzyme-linked immunosorbent assay
FAS	Fatty acid synthase
Fib-4	Fibrosis 4 score
GC	Gas chromatography
GC-MS	Gas chromatograph/mass spectrometry
GLP-1	Glucagon-like peptide 1
GMLVQ	Generalized matrix learning vector quantization
HbA1c	Glycated haemoglobin
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
HRA	(NHS) Health Research Authority
IL-6	Interleukin 6
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
LMS	LiverMultiScan (magnetic resonance imaging)
MHRA	Medicines and Healthcare Products Regulatory Agency
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid
NFS	NAFLD fibrosis score
NICE	National Institute for Health and Care Excellence
NHS	National Health Service
OCDEM	Oxford Centre for Diabetes, Endocrinology and Metabolism
OCMR	Oxford Centre for Clinical Magnetic Resonance Research
OGTT	Oral glucose tolerance test
OHB	β-hydroxybutyrate
QALY	Quality-adjusted life year
REC	Research ethics committee
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol (triglyceride)
TNFα	Tumour necrosis factor alpha
VLDL	Very low density lipoprotein

Chapter 1 Introduction and literature review

The material contained within this chapter has been adapted from papers which have either been published in the academic medical journal Frontiers in Pharmacology in a paper titled 'Non-invasive markers of liver fibrosis: adjuncts or alternatives to liver biopsy?' on which I am joint first author and led on sections of this work (1), and / or have been submitted for publication in the academic medical journal Endocrine Reviews in a paper titled 'Non-alcoholic fatty liver disease: Current concepts in pathophysiology, outcomes and management', on which I am second author and led on sections of this work (2).

1.1 Non-alcoholic fatty liver disease (NAFLD)

The global obesity and related type 2 diabetes (T2DM) epidemics are conditions which pose very significant morbidity and mortality burdens to both the individual and to society in general. Being part of the metabolic syndrome, they are closely related with ectopic fat deposition in the liver, known as non-alcoholic fatty liver disease (NAFLD).

NAFLD begins with simple steatosis (NAFL), defined as >5 % fat deposition within the liver. Steatosis may progress to inflammation, termed non-alcoholic steatohepatitis (NASH), and consequent fibrosis and cirrhosis. The presence of fibrosis – both early (stages F1-F2) and advanced (stages F3-F4, with F4 being cirrhosis) – is associated with increased morbidity and mortality and can lead to liver failure and development of hepatocellular carcinoma (HCC) (3).

The disease burden of NAFLD is manifested primarily through cardio-metabolic disease and its attendant complications, followed by cancer – both gastrointestinal and non-gastrointestinal – and then through liver failure and end-stage liver disease (3). Consequently, NAFLD should be viewed as a key component of the metabolic syndrome in addition to a liver disease and establishing a diagnosis of NAFLD is of high importance in risk stratifying patients.

1.2 Epidemiology of NAFLD

Current estimates suggest that NAFLD affects up to 30% of unselected populations worldwide, though this varies according to geographical region (4). Between 2005 to 2010 the global burden and prevalence of NAFLD is estimated to have rapidly increased from 15 to 25 % making it the commonest liver disease worldwide (4). Studies have also demonstrated that over 90 % of patients undergoing weight loss surgery have a diagnosis of NAFLD (5) and up to 70 % of patients with type 2 diabetes mellitus (T2DM) have NAFLD which confirm its close relationship to the metabolic syndrome.

Estimates also suggest that 6-30 % of patients with NAFLD have NASH and that NASH prevalence in the general population is between 1.5-6.45 % (4). Fibrosis progression has been estimated to be between 30-40 % in patients with NAFLD with an average annual fibrosis progression rate of 0.09% (4,6).

Between 2004 and 2009, there was a large annual increase of NAFLD related HCC cases of around 10 % in the USA (7). NAFLD-HCC patients were older, had a reduced life expectancy with more cardiovascular complications, and were more likely to die from their liver cancer compared with other HCC aetiologies. Similarly, in Europe, Dyson *et al.* reported a 10-fold increase in HCC associated with NAFLD between 2000-2010 with patients again being older on average and experiencing greater cardio-metabolic complications, though survival in this cohort was similar to other aetiologies of HCC (8). NAFLD-related HCC cases continue to rise with the NAFLD epidemic and HCC is currently the fourth leading cause of cancer mortality globally (9).

1.3 NAFLD and cardio-metabolic disease

The prevalence of NAFLD increases in high-risk groups, particularly those with obesity and T2DM. Studies in unselected populations have shown prevalence rates as high as 91 % in patients who were obese (BMI ≥30 kg/m^2) and 67 % in those who were overweight (BMI 25–30 kg/m^2) (10). The global increase in obesity, which commenced during the latter part of 20th century and continues today with rates of increase currently greatest in Asian populations, has been driven by rapid urbanisation and consequential lifestyle changes, increased calorie consumption of particularly calorie dense food, and over-nutrition.

In patients with T2DM, some studies report NAFLD prevalence to be as high as 60–70 % (11,12) with a recent meta-analysis of studies in patients with T2DM demonstrating a pooled NAFLD prevalence of 60 %, though this estimate ranged from 29.6-87.1 % (13). Data from the UK has suggested a prevalence of around 43 % (14). In a global meta-analysis of studies in unselected general populations, obesity and T2DM were present in 51 % and 23 % of those with NAFLD respectively (15). These data indicate the close relationships between obesity, T2DM and NAFLD, and NAFLD prevalence in these groups can be estimated as approximately double that of the general population (16).

Cardio-metabolic disease also appears to be an important driving factor in the development of HCC in patients both with and without cirrhosis (17,18). A recent study has shown that patients with NAFLD and T2DM were >19-times more likely to develop HCC and >6 times more likely to die from HCC compared to people without liver disease (19).

1.4 Cardio-metabolic outcomes in patients with NAFLD

Cardio-metabolic disease (CVD) and NAFLD have shared risk factors including insulin resistance, T2DM, obesity, hypertension and dyslipidaemia (20). Even after adjusting for conventional risk factors, NAFLD remains an independent risk factor for the development of CVD (15,21,22). Younossi *et al*. estimated the association between NAFLD and obesity as

51 %, T2DM 23 %, hyperlipidemia 69 %, hypertension 39 %, and metabolic syndrome 43 % (4).

Once CVD is established, there is strong evidence suggesting that patients with NAFLD have an increased severity of CVD burden (23). This may be related to the fact that patients with NASH have a greater degree of endothelial dysfunction, impaired left and right ventricular cardiac function (which can lead to heart failure), and carotid artery disease increasing the risk of cerebrovascular disease and stroke (24). NASH, through its pro-inflammatory state, also has systemic effects on various organ systems within the body, and likely exacerbates insulin resistance and worsens dyslipidaemia, so exacerbating development of CVD (25).

Body fat distribution (over and above obesity and measurements of BMI) is also an important consideration; patients with greater visceral adipose tissue, which has higher metabolic activity, are more likely to suffer from NAFLD and have a higher risk of developing cardio-metabolic complications (21).

A recent systematic review and meta-analysis has demonstrated that patients with NAFLD have a greater prevalence of coronary artery disease 7.5 % *vs.* 1.4 % and stroke 0.9 % *vs.* 0.2 % when compared to those without NAFLD. The risk of cardiovascular morbidity was 77 % higher compared to controls and cardiovascular mortality was 46% higher (26). Similarly, a further meta-analysis has shown that the presence of NAFLD is associated with a 64 % increase in the risk of both fatal and non-fatal cardiovascular events (25). These figures are similar to a recent retrospective study of patients with both NAFLD and T2DM in which the risk of cardiovascular events was 70 % higher, and all-cause mortality was 60 % higher when compared to those without liver disease (19).

In addition to these associations, patients with more advanced NAFLD have worse 10-year cardiovascular risk profiles (22), poorer cardiovascular outcomes, and are approximately 4-

times more likely to suffer a fatal CVD event and twice as likely to have a non-fatal CVD event (25,27).

NAFLD has a bidirectional relationship with T2DM; it is a risk factor for the development of T2DM with studies showing that those with NAFLD have a 1.5-2-fold increased risk of developing T2DM. Similarly, patients with T2DM are more likely to develop NAFLD (20,28). Indeed, the strongest predictor of fibrosis progression in those with NAFLD is the development of incident T2DM (29). The presence of NASH is also an important factor with these individuals having an additional up to 3-fold risk of developing T2DM (30). In turn, the presence of T2DM worsens the course of liver fibrosis, is more likely to lead to the development of NASH, is a predictor of HCC development and is associated with increased all-cause and cardiovascular mortality (23). Finally, the combination of both T2DM and NASH together leads to even worse outcomes, both with respect to cardiovascular disease and liver-related outcomes.

Patients with NAFLD are also at increased of conditions related to cardiovascular disease and T2DM. In particular, the prevalence of chronic kidney disease (CKD) in those with NAFLD has been shown to be as high as 55 % in some studies even after adjustment for other causal factors including T2DM and hypertension. Those patients with NASH and those with advanced fibrosis have both higher prevalence and incidence of CKD (20,31,32).

1.5 Liver related outcomes in patients with NAFLD

Liver failure, including end-stage liver disease is the third clinical scenario through which NAFLD exerts its morbidity and mortality. Traditionally, it has been assumed that NAFL without accompanying NASH was an indolent disease with relatively slow progression compared to NASH which progresses faster to advanced fibrosis. Indeed, it is NASH which is the predominant form of NAFLD that progresses to advanced fibrotic disease with

estimates suggesting that 10-15 % of those with NASH progress on to cirrhosis with higher rates of decompensated liver disease and HCC development (15,33). Furthermore, the presence of T2DM in patients with NASH is an independent predictor of liver-related mortality and similarly, advanced fibrosis in those with NASH is an independent risk factor for predicting mortality (15).

Nevertheless, recent studies have shown that a significant proportion of those with NAFL do progress to advanced fibrosis with McPherson *et al.* reporting that 44% of patients progressed to NASH, 37% progressed to liver fibrosis and 22% to advanced liver fibrosis (29). Though this means that the majority of those with NAFL have stable disease and do not progress, *Singh et al*. in their meta-analysis showed a rate of progression in those with NAFL of 1 fibrosis stage approximately every 14 years, and double this rate for those with NASH of 1 fibrosis stage every 7 years (34).

Previously it had been thought that only those with advanced liver fibrosis were at risk of increased all-cause mortality (27,35). However, there is now convincing data indicating that even those patients with early (F1) fibrosis have increased all-cause mortality compared to reference populations without fibrosis (36). Furthermore, liver-specific mortality rises exponentially with each increase of fibrosis stage (36,37). Though still a keenly debated issue, this more recent data also suggests that fibrosis stage, irrespective of the presence of NASH, appears to be the most important determinant of outcomes in patients (27,38,39) and that the presence of other risk factors, including but not limited to age, T2DM and smoking appear to accelerate fibrosis progression and development of liver failure. As expected, patients with cirrhotic NAFLD have worse liver-related and cardiovascular outcomes compared to those patients without cirrhosis; liver-related mortality is estimated at 24 % in F3 compared with 59 % in those with cirrhosis (F4) (36).

1.6 NAFLD and liver transplantation

NAFLD results in the need for liver transplantation through hepatic decompensation as a consequence of cirrhosis, which affects 15-25 % of those with NASH, and or the development of HCC in those with established cirrhosis (4). Since the mid-2000s, liver transplantation rates for NASH have risen to make it the second leading cause of liver transplantation worldwide, and it is predicted to be the commonest cause for liver transplantation within the next few years (40,41).

The age of those undergoing liver transplantation for NASH has also fallen significantly over the last 15 years, mirroring the upward trend of overall NAFLD incidence which increased over a ten-year period, particularly in the young (<45 years), where incident cases in this group increased by 7.5 % each year (42). Analysis of transplant registrants in the United Network for Organ Sharing Register (1995-2015), which profiled over 180,000 individuals, showed a marked upward trend in NASH with, and without HCC since 2005. Those aged between 35-55 years had the greatest increase in registration rates for liver transplantation (43). The authors termed this the 'adipose wave effect' as it is line with data that shows obesity and metabolic diseases are increasingly affecting people at younger ages. This shift in epidemiology towards younger populations is a stark reminder that the global burden of NAFLD and its complications including HCC, liver failure and cardiovascular events is only set to increase in the foreseeable future.

1.7 Pathophysiology of NAFLD

1.7.1 Diet and NAFLD

A westernised diet with high calorific intake of high fat, high sugar and processed foods is linked with the development of NAFLD. Indeed, patients with NAFLD have a higher daily total energy intake when compared to matched healthy controls (44,45). Mediterranean diets, which generally contain lower saturated fat and cholesterol, higher monounsaturated

fatty acids and more complex carbohydrates and fibre, are associated with lower rates of NAFLD (46–49). Fructose, which is a major component of dietary sweeteners, is linked with NAFLD (50–52).. Its consumption has increased significantly during the rise of the obesity pandemic and it is estimated to constitute around 15 % of the energy consumed as part of a westernised diet (53). Additionally, fructose appears to cause a greater disruption to lipid homeostatic pathways within the liver (see section 1.7.3) compared to glucose which in turn may promote the development of hepatic steatosis (54–58) and NASH (59,60).

1.7.2 Genetic predisposition

Not all subjects who are obese suffer from its associated cardio-metabolic complications including NAFLD and genetic factors are believed to play a significant role in both the predisposition to and development of NAFLD as well as to its progression and outcomes. Indeed, first degree relatives of patients with NAFLD have up to 3-times greater risk of developing NAFLD compared to the general population (61). Furthermore, 'lean NAFLD' (NAFLD occurring at a BMI <25 kg/m^2) is a well-recognised clinical entity with prevalence estimated at up to 20 % depending on geography (62,63), and body fat distribution – in particular greater visceral adipose tissue – is linked with NAFLD (21).

Several gene variants have been identified from genome-wide association studies that show an association with NAFLD development and progression. These include the PNPLA3 polymorphism (64) where there is an isoleucine to methionine substitution at position 148 (rs738409 C>G encoding for PNPLA3 I148M) which is thought to affect triacylglycerol (triglyceride, TAG) turnover (65) and regulate the activity of hepatic stellate cells (HSC) which promotes fibrosis (66). A gene dosage effect has also been noted with severity of both NASH and fibrosis increased in homozygotes as well as an increased risk of HCC. The polymorphism is most common in those of Hispanic ethnicity and lower in those of European or African ethnicity (67). Interestingly, those with the polymorphism may also respond more

favourably to interventions. As such, PNPLA3 is now well recognised to be a modifier of disease outcome across the spectrum of NAFLD (67).

Variations in the TM6SF2 gene, which involves a guanine to adenine substitution at position 167 (rs58542926 E>K encoding for TM6SF2 E167K) is also well described in NAFLD. Presence of this polymorphism promotes steatosis by impairing secretion of very low-density lipoprotein (VLDL) (68) and has been demonstrated to increase the risk of advanced fibrosis by twofold (67). Of further note, this gene variant also appears to confer some cardiovascular protection by lowering serum lipid and related metabolite levels potentially at the expense of the liver – the so called “TM6SF2 Catch-22” paradigm (67,69). Most of the detailed studies on the TM6SF2 gene have been undertaken in ethnically European populations and further study on geographical prevalence of TM6SF2 variants, as well on HCC risk is required.

Recently, polymorphisms within 17β-hydroxysteroid dehydrogenase type 13 (HSD17B13) have also been described in subjects with NAFLD, NASH and liver fibrosis (70,71). Other genetic polymorphisms associated with NAFLD include MBOAT7 and GCKR and work is ongoing to better understand natural history and disease progression and how this new genetic knowledge may assist the identification of novel therapeutic targets (72).

1.7.3 Dysregulated lipid and carbohydrate metabolism

In summary, NAFL and NASH result where there is excess influx or reduced disposal of free fatty acids, processes which are governed by complex metabolic pathways, controlled in combination centrally by the brain, locally within the gut and the liver and peripherally within fat and muscle.

1.7.3.1 Fat accumulation within the liver (liver steatosis)

Liver steatosis results from an imbalance of lipid handling and homeostasis within the liver and in particular, the production and disposal of free fatty acids (FFAs) also known as non-esterified fatty acids (NEFA) (See Figure 1.1). FFAs are made available in the liver either through dietary intake, from the breakdown of peripheral lipid stores, or via synthesis from non-lipid sources (de novo lipogenesis, DNL, primarily from the carbohydrates fructose and glucose). In NAFLD, there is excess dietary fat intake. Additionally, DNL appears to be upregulated (73,74), a process at least in part driven by insulin which appears to selectively support DNL whilst failing to suppress hepatic gluconeogenesis (75,76).

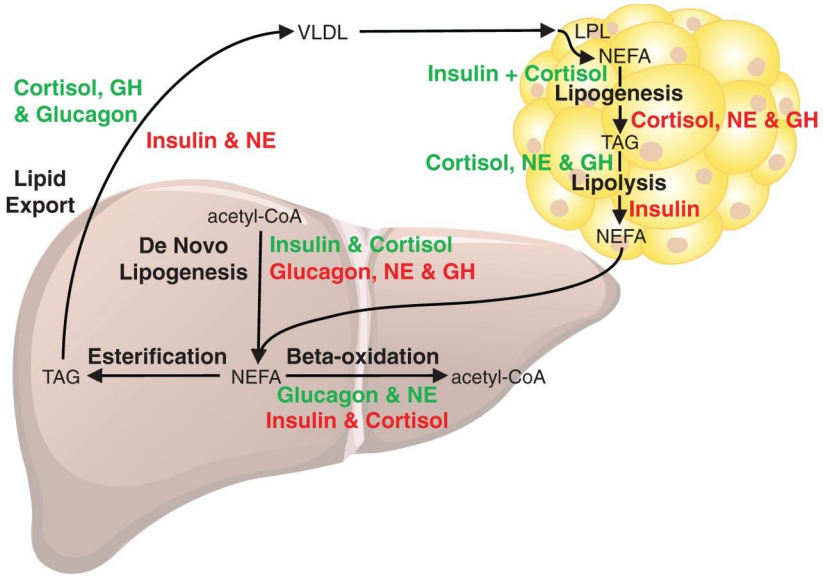
FFA disposal from the liver is either via beta oxidation to generate energy or esterification to TAG) which is either stored within the liver or transported to peripheral lipid stores as very low-density lipoprotein (VLDL). The increased lipid burden in NAFL leads to the upregulation of mitochondrial activity (77) and over time this increased burden can lead to liver injury through mitochondrial dysfunction and oxidative stress (78).

Figure 1.1: Lipid and glucose handling within the liver and the effect of glucoregulatory hormones on homeostasis.

Figure and caption reproduced and adapted from Geisler and Renquist (79).

Effect of glucoregulatory hormones on hepatic lipid metabolism: Ingested glucose is metabolised via the tricarboxylic acid (TCA) cycle to a-CoA. Ingested fat (as chylomicrons) is transported to and enter the liver as TAG. Glucagon, cortisol, growth hormone (GH) and norepinephrine (NE) all act counter-regulatory to insulin to affect glucose homeostasis, yet have divergent effects on lipid homeostasis. Hormone signalling can induce an increase in hepatic lipid accumulation through increased adipose tissue lipolysis and hepatic lipid clearance, increased de novo lipogenesis, decreased beta-oxidation or decreased lipid export as VLDL particles. Hormones written in green text activate the indicated pathway, while hormones in red text inhibit the pathway.

NEFA: Non-esterified fatty acid, TAG: Triglycerol, LPL: lipoprotein lipase, VLDL: very low density lipoprotein.



1.7.3.2 Adipose tissue dysfunction

Adipose tissue is a metabolically active organ which acts as a biological buffer to protect the body from over-nutrition. Lipid storage in adipose tissue is tightly regulated by insulin, which as an anabolic hormone, prevents the breakdown of fat (lipolysis) and production of glucose in the liver (gluconeogenesis) and serves to reduce the liver's exposure to pro-inflammatory FFAs (80). Over-nutrition, obesity and diabetes result in a pro-inflammatory state both systemically, and within adipose tissue, which leads to the release of cytokines and chemokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (80,81) and consequently to impairment of usual homeostatic and metabolic regulatory pathways. In insulin resistant or diabetic states, the inhibition of lipolysis by insulin is reduced leading to increased FFA flux into the liver. Unlike in adipose tissue, the liver is unable to safely store the excess fat being returned to it, and with its usual metabolic regulatory pathways overwhelmed, therein starts a self-perpetuating cycle of initially fat build up closely followed by inflammation (NASH).

Adipose tissue dysfunction, medicated by pro-inflammatory cytokines, also leads to the reduced production of adiponectin, an insulin-sensitising adipokine. Adiponectin has wide-

ranging hepato-protective effects increasing FFA oxidation and decreasing FFA influx, gluconeogenesis and DNL (82–84). It also has anti-inflammatory properties in the liver through suppression of cytokines including of TNF-α and IL-6 (85,86), and protects against fibrosis through reduced activation and proliferation of hepatic stellate cells (87–89). Circulating adiponectin levels are reduced in NAFLD and correlate with the degree of hepatic steatosis, necro-inflammation, and fibrosis (90–92).

1.8 Endocrine disease, glucocorticoid metabolism and NAFLD

Endocrine diseases in addition to diabetes, including growth hormone deficiency, hypothyroidism, polycystic ovarian syndrome, hyperaldosteronism and hypogonadism are all associated with NAFLD (79,93,94). NALFD also has a sexually dimorphic prevalence, being more common in men than women. Oestrogens are thus thought to play a role in NAFLD development. Adipose tissue is a significant source of oestrogen production and metabolism (95) and oestradiol is thought to be protective against NAFLD (96).

Furthermore, dysregulated glucocorticoid (GC) metabolism is implicated in the pathogenesis of NAFLD. Both endogenous GC excess – Cushing's disease, and exogenous GC excess, for example glucocorticoid replacement therapy, are associated with NAFLD (97). Estimates suggest that around 20 % of patients with Cushing's syndrome have a co-existent diagnosis of NAFLD though the true prevalence is uncertain and may be higher (98,99).

Changes to GC metabolism have been described in small numbers of individuals and relate to cortisol and its metabolites (100,101) and the enzymatic pathways that regulate cortisol bioavailability within the liver and its ability to bind and active the GC receptor. In particular, the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which regenerates cortisol (F) from inactive cortisone (E), and the A-ring reductase family of enzymes – 5α and 5β reductase (5αR/5βR) – which inactivate cortisol to tetrahydrocortisol metabolites (THF/5αTHF), are the key enzymes involved in cortisol metabolism. Inhibition of these

enzymes has been shown in a number of studies to influence the accumulation of liver fat and metabolic syndrome. 11β-HSD1 inhibition reduces liver fat accumulation (102), whilst 5αR inhibition results in accumulation of liver fat (103).

1.9 Diagnosis of NAFLD

1.9.1 Clinical features

NAFLD is a diagnosis of exclusion. Determination of NAFL requires the presence of intrahepatocellular lipid droplets in >5 % of hepatocytes. Patients are most often asymptomatic though some may experience generalised fatigue or abdominal discomfort, particularly over the anatomical location of the liver in the right upper quadrant of the abdomen.

Diagnosis is often made after an incidental finding of liver abnormalities, for example mildly elevated liver biochemistry on blood testing or features of steatosis being noted on radiological imaging such as ultrasound (US), computed tomography (CT) or magnetic resonance (MR) imaging. The presence of one or more cardio-metabolic risk factors, such as obesity, T2DM or impaired glucose tolerance, hypertension and dyslipidemia is important, as is the absence of other causes of hepatic steatosis and chronic liver diseases (33,104). In addition, the absence of excessive alcohol consumption is required as determined in the clinical history, which is defined by the European Association for the Study of the Liver (EASL) as ≥20 g/day for a woman or ≥ 30g/day for a man (104), with the UK definition of 1 unit of alcohol being 8 g (www.nhs.uk/live-well/alcohol-support/calculating-alcohol-units).

1.9.2 Clinical investigations

In primary care, general practitioners will most often as a first line investigation perform liver function tests including alanine aminotransferase (ALT) and / or aspartate aminotransferase (AST), as well as a liver ultrasound, if not already performed, to investigate for potential liver disease and if these raise concerns, will refer patients to secondary care clinics.

It is important to note however that measurement of ALT is neither a specific nor sensitive screen for NAFLD and a 'normal' ALT result should be treated with caution. For example, one recent study in patients with T2DM demonstrated that 50% of patients diagnosed with NAFLD had normal plasma aminotransferases levels (105) and further work to increase awareness in this regard is required both in primary and secondary care settings (106).

In secondary care, further investigations will often be performed to exclude other causes of liver disease. As such, blood testing should include a full blood count and bone profile, as platelet count an serum albumin are important markers of liver synthetic function; liver biochemistry; renal function; a screen for diabetes (fasting blood glucose or glycosylated haemoglobin (HbA1c)); lipid profile; serum ferritin and transferrin saturations to assess for iron overload; thyroid function; hepatitis B and C serology; liver autoantibodies (anti-nuclear antibody, anti-mitochondrial antibody, anti-smooth muscle antibody, anti-liver kidney microsomal antibody); immunoglobulins (A, G, M); and α-1 antitrypsin. Other causes which may need to be excluded include dysregulated copper metabolism (serum caeruloplasmin) and coeliac disease.

1.10 Disease staging and risk stratification in NAFLD

1.10.1 The importance of staging and risk stratification for prognostication

As all cause disease outcomes including both morbidity and mortality from NAFLD are related to the stage of liver disease, with liver-specific mortality rising exponentially with each increase of fibrosis stage (36,37) and those with advanced fibrosis suffering from the

greatest cardio-metabolic morbidity and mortality burdens (3), establishing the initial diagnosis of NAFLD followed by thorough assessment to allow accurate staging and risk stratification is of crucial importance.

In this regard, determination of the stage of liver fibrosis is the most important factor governing risk stratification and prognostication. Determining the presence of NASH is also of importance, though its use as a prognostic factor is less clear and under debate (27,37,38).

Accurate staging of NAFLD has high importance for two further reasons. Firstly, it allows for enhanced disease monitoring (progression or regression), for the development of clinical complications and for the personalisation of therapy thereafter where appropriate, for example with targeted diabetes or cardiovascular therapy. Secondly, it assists clinicians and health commissioners to better plan services, based on the needs of their local populations. Due to the high prevalence of NAFLD, it is not possible to manage all patients in secondary care. Thus, accurate clinical staging and risk stratification helps identify those with advanced liver disease and most at risk of complications so that this high risk population can be better targeted and managed appropriately.

1.10.2 Liver biopsy – the current gold standard disease staging tool

Liver biopsy is the gold standard diagnostic tool with which to risk stratify and stage NAFLD after the initial diagnosis has been made as it allows for both direct assessment of liver inflammation and fibrosis. In this regard, various scoring systems have been proposed to characterise and grade liver biopsy tissue on histology. These include the NAFLD Activity Score (NAS) to quantify histological features associated with a diagnosis of NASH (107), and the NASH CRN system used to determine fibrosis stage and which is used to distinguish early fibrosis (stages F1-2) from advanced (clinically significant) fibrosis (stages F3-F4), with F4 fibrosis being termed cirrhosis (108).

1.10.2.1 Limitations of liver biopsy

Whilst liver biopsy can be helpful in elucidating the cause of liver disease where aetiology is uncertain, this is not the primary purpose of performing a biopsy in patients with suspected NAFLD. Furthermore, the histological features of biopsy tissue are indistinguishable between cases of alcohol related liver disease (ALD) and NAFLD.

Additionally, liver biopsy is an invasive procedure and is associated with significant clinical risks and complications including pain, bleeding and infection (109). Estimates suggest rates of serious complications arising from liver biopsy, including haematoma, infection and pain, are up to 2 % (110).

There are also resource implications when performing liver biopsy; a range of healthcare professionals are required to arrange and perform the biopsy, analyse the tissue and discuss the findings and include hepatologists, interventional radiologists and histopathologists. Furthermore, biopsy is performed in the secondary care environment and so requires a day case hospital admission with allocation of a bed to allow for post-procedure monitoring and thus requires the need and support of allied healthcare professionals.

It is also important to note that biopsy samples tissue from only one region of the liver and thus does not allow for a 'whole liver' assessment and so there remains a significant possibility for sampling variability and technical error as well as inter- and intra- observer variation on reporting of samples (111). Indeed, whilst the various histological scoring systems outlined assist histopathologists to grade tissue, scoring remains subjective, different reporters may report and grade the same sample of tissue differently.

Finally, due to its invasive nature and resource implications, liver biopsy is not well suited for use as a disease monitoring tool after instigation of therapy and it is difficult to justify serial biopsies for disease monitoring. Furthermore, from personal experience, it is common for

patients to report negative experiences and thus reluctance to consent for repeat biopsy should the need arise.

1.11 Non-invasive risk stratification tools for NAFLD

There are many non-invasive biomarkers investigated and proposed for NAFLD which may help reduced the need for liver biopsy, though as yet, none have fully replaced liver biopsy. An ideal non-invasive test would be quick to perform and analyse, inexpensive, robust, sensitive, specific and reproducible. It would further ideally need to distinguish between and track the various stages of fibrosis, and determine the presence of NASH.

In general, most of the existing biomarkers described in the context of NAFLD work best to predict advanced fibrosis when compared to the gold standard of liver biopsy. Importantly, some also have an 'indeterminate range' where the tests are unable to risk stratify disease stage which limits their utility in clinical practice.

More generally, it is also important at this point to define what is meant by a biomarker and how it may be used in practice. In this regard the BEST (Biomarkers, EndpointS, and other Tools) Resource Glossary (112) defines a biomarker as a:

> *"defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions"*

Furthermore, the BEST Resource and Glossary outline seven distinct biomarker categories – susceptibility/risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic/response, and safety – and go on to outline how the biomarker category should be further qualified to describe the specific context in which it will be used (context of use), for example in drug development, clinical care or other research.

1.11.1 Clinical biomarkers

As outlined in sections 1.3-1.4, clinical parameters which help risk stratify patients with NAFLD include presence and / or adequate control of T2DM and other features of metabolic syndrome (113) including obesity, as well as a smoking history, age, and concurrent therapy including statin use (29,37).

1.11.2 Serological biomarkers

Serological or blood based markers are either biochemical or haematological and can be broadly divided into two classes. Class I markers are 'direct serum markers' of liver fibrosis which reflect extracellular matrix (ECM) turnover. Fibrotic changes in the liver are derived from hepatic stellate cells (HSCs) and myofibroblasts (1). Upon liver injury, release of cytokines leads to the activation of HSCs causing liver inflammation, which leads to change in tissue architecture and the ECM.

Class I markers may be enzymatic, collagen related, glycoprotein and matrix-metalloproteinase related and glycosaminoglycan related (1) (See Table 1.1). Relatively few class I markers are used in routine clinical practice as they often do not correlate with whole tissue or body function, and consequentially, there availability is limited with higher turnaround times and costs compared to class II markers. Furthermore, class I markers are not all derived from the liver and may not be liver specific and thus serum measurements may reflect 'whole-body' activity, for example systemic inflammation, rather than being liver-specific.

Table 1.1: Examples of Class I (direct) serum non-invasive biomarkers for liver fibrosis

Table adapted from Chin et al (1).

Metalloproteinases (MMPs)	*Collagen Type IV, VI*
Tissue inhibitors of metalloproteinases (TIMPs)	*Transforming growth factor (TGF)-β*
Hyaluronic acid (HA)	*Microfibril-associated protein 4 (MFAP4)*
Laminin	*N Glycans- reflect regenerative nodules*
Fibronectin	*Procollagen type I carboxy-terminal peptide (PINP)*
Chitinase-3-like protein 1 (CHI3L1 or YKL-40)	*Procollagen type III amino-terminal peptide (PIIINP)*

Class II markers of liver fibrosis are termed 'indirect' markers as they are derived from changes that occur in liver function. As these often correlate with signs and symptoms of liver disease, their use in clinical practice is greater which in turn means they have better availability and costs are lower. The liver performs a broad range of activities to includes thermostasis, energy balance, carbohydrate, fat and protein metabolism, production of blood clotting factors and bile acids, iron and haemoglobin metabolism, immune balance, and drug clearance. As such, the range of class II markers is wide. In routine clinical practice, the broad categories of tests can be categorised as follows (1):

- **Liver enzymes**, including alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT)
- **Markers of synthetic function**, such as prothrombin time (PT / INR), bilirubin, haptoglobin, albumin, Apolipoprotein A1, α2-macroglobulin, ceruloplasmin, transferrin and hepcidin
- **Other indirectly measured markers**, such as platelet count, α1-antitrypsin, ferritin, and certain adipokines (adiponectin and leptin)

1.11.3 Combination or panel biomarkers

Combination or panel biomarkers incorporating various clinical, serological and associated parameters and using varying degrees of complexity, can help to improve the sensitivity and specificity of non-invasive assessment of liver fibrosis. These have been validated in and reviewed for the various aetiologies of liver fibrosis (1,114,115).

Serum AST and ALT, are important components which are used by a number of combination biomarkers and indeed the ratio of AST to ALT is one such biomarker (116). The Fib-4 score which uses age and platelet count in addition to AST an ALT (117) and the NAFLD Fibrosis Score (NFS) which includes platelet count, albumin concentration, BMI and the presence or absence of T2DM or impaired fasting glucose (118), and the BARD score which includes BMI and presence or absence of T2DM or impaired fasting glucose (119) are further examples which use class II biomarkers in combination with clinical markers. Calculation of these scores is determined as follows:

- Fib-4 (Fibrosis-4 score) = age × AST (IU/L) / platelet count ($\times 10^9$/L) × $\sqrt{}$ALT (IU/L)
- NAFLD fibrosis score = −1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m^2) + 1.13 × Impaired fasting glucose or T2D (yes = 1, no = 0) + 0.99 × AST/ALT ratio −0.013 × platelet count ($\times 10^9$/L) −0.66×albumin (g/dL)

Both the Fib-4 score and the NFS use two thresholds; a lower value below which there is a high negative predictive value (NPV) to exclude the presence of advanced fibrosis, and a higher value above which the specificity and positive predictive value (PPV) is maximised to predict advanced fibrosis (120). For predicting advanced fibrosis (stages 3-4), a Fib-4 score of <1.3 has a 90 % NPV and a score of >2.67 has an 80 % PPV (121). For the NFS, the low cut-off score of (<−1.455) has an NPV of 93 % and the high cut-off score (>0.676) has a PPV of up to 90 % (122).These scores also have an indeterminate range, have generally modest PPV, and may be confounded, for example by age which can limit utility (123).

More complex combination and often proprietary panel markers have been developed which usually incorporate both class I and class II serological markers in addition to mathematical algorithms. The Enhanced Liver Fibrosis (ELF) score, is one such marker which includes assessment of hyaluronic acid, procollagenase 3 and metalloproteinase 1, which has been recommended by the UK National Institute for Health and Care Excellence (NICE) and has been estimated to have a diagnostic accuracy (AUROC) for advanced fibrosis in NAFLD of 93% (124) with a PPV for advanced fibrosis of 80-87 % and an NPV of 96-98 % which is very similar to the performance of the NFS (125). Other panel biomarkers include the Fibrotest/Fibrosure®, Fibrometer® and Hepascore®. Please see Table 1.2 for a detailed summary of the performance of non-invasive markers of liver fibrosis.

Combination markers often come at an increased cost and availability can be limited even with the NICE recommended ELF test. Whilst they show a general improvement over simpler combination markers, concrete evidence of this benefit and longer term performance remain limited. For example, the FibroTest panel performed no better than the NAFLD Fibrosis Score when compared to histological fibrosis staging, for diagnostic accuracy in one study of 574 patients (126), and other studies have shown that the Fib-4 score and aminotransferase to platelet ratio index (APRI) have value in both evaluation of liver fibrosis and in prognosticating liver disease (1).

Table 1.2: Non-invasive serum biomarkers of liver fibrosis.

Table reproduced from Chin et al. (1)*. For references contained see Chin et al. full paper appended in Chapter 6,*

Index	Parameters	Year	Disease	*n*	AUROC for advanced fibrosis	Sens.	Spec.	NPV	PPV	Lead author
AST/ALT Ratio (De Ritis /Sheth)	AST/ALT	1998	HCV	139	–	53+	100+	81+	100+	Sheth et al., 1998
		2010	NAFLD	145	0.83	74	78	93	44	McPherson et al., 2010
		2015	PBC	137	0.59	–	–	–	–	Umemura et al., 2015
		2000	HCV	151	–	47+	96+	88+	74+	Park et al., 2000
BARD	BMI, AST, ALT, DM	2008	NAFLD	827	0.81	–	–	–	–	Harrison et al., 2008
		2010	NAFLD	145	0.77	89	44	95	27	McPherson et al., 2010
		2011	NAFLD	138	0.67	51	77	81	45	Ruffillo et al., 2011
		2016	NAFLD^	1038	0.76	74	66	–	–	Sun et al., 2016^
Bonacini-index	ALT/AST-ratio, INR, platelet count	1997	HCV	79	–	46	98	–	–	Bonacini et al., 1997
FIB-4	Platelet count, AST, ALT, age	2006	HCV/HIV	830	0.74–0.77	67	71	89	38	Sterling et al., 2006
		2010	NAFLD	145	0.86	85	65	95	36	McPherson et al., 2010
		2009	NAFLD	541	0.80	52	90	–	–	Shah et al., 2009
		2016	NAFLD^	1038	0.85	84	69	–	–	Sun et al., 2016^
		2015	PBC	137	0.71	–	–	–	–	Umemura et al., 2015
Fibrometer test	Platelet count, prothrombin index, AST, α2-macro-globulin, hyaluronic acid, urea, age	2005	HCV/HBV	383	0.89**	81**	84**	77**	86**	Cales et al., 2005
Fibrometer A	Prothrombin index,' α2 macroglobulin, hyaluronic acid, age	2005	ALD	95	0.96**	92**	93**	83**	97**	Cales et al., 2005
		2008	ALD	103	0.88	–	–	–	–	Nguyen-Khac et al., 2008
		2009	ALD	218	0.83	–	–	–	–	Naveau et al., 2009
Fibrotest (FT)	Haptoglobin, α2-macro-globulin, apolipoprotein A1. GGT, bilirubin, age, gender	2001	HCV	339	0.87	75	85	80	80	Imbert-Bismut et al., 2001
		2014	HBV^	2494	0.84**	61**	80**	–	–	Salkic et al., 2014^
		2009	ALD	218	0.83	–	–	–	–	Naveau et al., 2009
		2008	ALD	103	0.8	–	–	–	–	Nguyen-Khac et al., 2008
		2006	NAFLD	267	0.81	92	71	98	33	Ratziu et al., 2006
		2003	HCV	352	0.73	–	–	–	–	Poynard et al., 2003
Forns-index	Age, platelet count, GGT, cholesterol	2002	HCV	476	0.81–0.86**	94**	51**	96**	40**	Forns et al., 2002
Hepascore	Bilirubin, GGT, Hyaluronic acid, α2-macroglobulin, age, gender	2005	HCV	221	0.9–0.96	74–81	88–95	95–98	–	Adams et al., 2005
		2009	ALD	218	0.83	–	–	–	–	Naveau et al., 2009
		2008	ALD	103	0.83	–	–	–	–	Nguyen-Khac et al., 2008
Hui	Body mass index (BMI), platelet count, serum albumin, and total bilirubin	2005	HBV	235	0.79	88	50	92	38	Hui et al., 2005
Leroy-score	PIIINP, MMP-1	2004	HCV	194	0.88	58	92	–	91	Leroy et al., 2004
NAFLD Fibrosis Score (NFS)	Age, BMI, platelets, albumin, AST/ALT, IFG /diabetes	2007	NAFLD	733	0.82–0.88	77–82	71–77	88–93	52–56	Angulo et al., 2007
		2010	NAFLD	145	0.81	33–78	58–98	86–92	30–79	McPherson et al., 2010
		2016	NAFLD^	1038	0.84	77	70	–	–	Sun et al., 2016^
Fibrospect-II	Hyaluronic acid, TIMP-1, α2-macroglobulin	2004	HCV	696	0.82–0.83**	77–83**	66–73**	–	74**	Patel et al., 2004
PGA-index	Prothrombin time, GGT, apolipoprotein A1									
		1993	Mixed	169	–	91+	81+	–	–	Teare et al., 1993
		2008	ALD	103	0.84	–	–	–	–	Nguyen-Khac et al., 2008
PGAA-index	Prothrombin time, GGT, apolipoprotein A1, α2-macroglobulin	1994	ALD	525	–	79+	89+	–	–	Naveau et al., 1994
		2008	ALD	103	0.86	–	–	–	–	Nguyen-Khac et al., 2008
Pohl score	AST/ALT-ratio, platelet count	2001	HCV	221	–	41	99	93	85	Pohl et al., 2001
ELF score	Hyaluronic acid, TIMP-1, age, MMP-3	2004	HCV	496	0.77	95	29	95	28	Rosenberg et al., 2004
		2004	NAFLD	61	0.87	89	96	98	80	
		2004	ALD	61	0.94	93	100	86	100	
		2008	NAFLD	192	0.90	80	90	94	71	Guha et al., 2008
		2008	PBC	161	0.75	–	–	–	–	Mayo et al., 2008
		2014	Mixed^	1645	0.87	78	76	–	–	Xie et al., 2014^
SHASTA	HA, AST, albumin	2005	HCV-HIV	95	0.87	50	94	83	76	Kelleher et al., 2005
Fibrosis probability-index, FPI	Age, AST, cholesterol, insulin resistance (HOMA), past alcohol intake	2004	HCV	302	0.77–0.84**	96**	44**	93**	61**	Sud et al., 2004
APRI score	AST, platelet count	2003	HCV	270	0.8–0.88**	41–91**	47–95**	61–88**	64–86**	Wai et al., 2003
		2010	NAFLD	145	0.67	27	89	84	37	McPherson et al., 2010
		2008	ALD	103	0.43	–	–	–	–	Nguyen-Khac et al., 2008
		2015	PBC	137	0.84	–	–	–	–	Umemura et al., 2015
		2008	HBV	264	0.86**	87**	66**	81**	74**	Shin et al., 2008
Zeng	alpha2-macroglobulin, age, gamma glutamyl transpeptidase, and hyaluronic acid	2005	HBV	372	0.77–0.84**	35–95**	44–95**	51–86**	70–91**	Zeng et al., 2005

ALT, alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio; GGT, γ-glutamyltransferase; PIIINP, N-terminal propeptide of type III procollagen; TIMP, tissue inhibitors of metalloproteinases; MMP, matrix metalloproteinases.
***Values are for prediction of significant fibrosis.*
+Values are for prediction of cirrhosis.
^Metaanalysis.

1.11.4 Imaging biomarkers

1.11.4.1 Hepatic steatosis

Non-invasive quantification and grading of liver steatosis can be achieved through the use of imaging modalities assessment such as MR-Spectroscopy (MRS), MR-Imaging (MRI proton density fat fraction (MRI-PDFF)) or the Controlled Attenuation Parameter (CAP) which is based on the transient elastography (127).

MRS is the gold standard for fat quantification. Its advantages include allowing assessment of the entire liver without limitation by acoustic windows or overlaying fat, which helps reduce sampling error, and it can provide information on anatomy. It does not involve ionising radiation and thus also allows for repeated assessment. Due to these advantages, MR is being used more frequently as a surrogate end-point in NAFLD clinical trials (128,129). MR techniques also allow for the gold standard quantification of liver iron and have shown very good inverse association with biochemically quantified hepatic iron levels (130,131).

1.11.4.2 Hepatic Elastography

The accurate staging of fibrosis using imaging modalities is more challenging and involves assessment of the liver parenchyma. Elastography is based around the concept of measuring vibration-induced shear waves through liver tissue. This includes both generating a pulse or vibration and a way to measure the impact of this pulse (reflectivity) within the liver. Both ultrasonographic and magnetic resonance techniques have been developed in this regard.

Vibration-controlled transient elastography (TE, FibroScan®) is an ultrasound based technique and involves placing a probe against the skin between the ribs overlying the right lobe of the liver (132). It has been widely validated; from the various studies undertaken

using TE, AUCs for prediction of advanced fibrosis or cirrhosis (F0-2 *vs.* F3-4) have been reported to vary between 0.83-0.93 (133), though the sensitivity and specificity vary according to the cut-off values used and can be set based on the clinical needs of the service in which it is employed. It's diagnostic performance also decreases in those with high BMIs. Other ultrasound based techniques include Acoustic Radiation Force Imaging (ARFI) and 2-dimensional shear wave elastography which are applications integrated on ultrasound scanning machines. These techniques have increasingly been validated with similar performance characteristics, but on fewer patients than TE (133).

Magnetic resonance elastography (MRE) also requires a probe placed adjacent to the patient's liver which is connected to a pneumatic active driver outside the scanner room which generates the mechanical waves required to assess liver scarring. This is additional dedicated hardware that needs to be installed onto existing MR scanners which require standardisation of protocols across centres. MRE has been validated in patients with NAFLD (134) with high diagnostic accuracy for advanced fibrosis (pooled sensitivity 86%, specificity 91%) in a recent comprehensive meta-analysis (135), although failure rate again increases with increasing BMI (136).

1.11.4.3 Multi-parametric Magnetic Resonance imaging techniques

Whilst ^{1}H-MRS (137) and MRI-PDFF (138,139) are able to sensitively quantify hepatic steatosis, their use in risk stratification is limited (140). Multi-parametric MR (MMR) imaging techniques however show greater promise to aid the risk stratification process through the ability to provide a surrogate assessment of liver fibrosis. MMR uses functional imaging techniques to complement the principle T1 (longitudinal relaxation time) and T2 (transverse relaxation time) measurements which are primarily used to assess anatomical structure. These techniques may be used in conjunction with MRI-PDFF to provide an assessment of liver fibrosis and inflammation and steatosis as per the LiverMultiScan diagnostic aid

pioneered by Perspectum Diagnostics (https://perspectum-diagnostics.com). To provide a surrogate assessment of liver fibrosis, algorithms are applied to the data captured by MMR which allow assessment of an iron-corrected T1 (cT1) (141,142). Furthermore, cT1 has been shown to correlate inversely with disease severity in NAFLD (143) and MMR has been shown to provide consistent and reproducible results (144). As this technique is a computational software based analysis, sequences can be predefined, and can be incorporated into existing hardware and scanning protocols. This removes the need to purchase additional hardware. Additionally, this technique can be employed across different scanner makes, models and field strengths and is delivered via a cloud based quality control system which allows remote monitoring and enhances standardisation and method improvement processes.

The place of these promising MR techniques in clinical practice is, as yet, unclear as even though reproducibility is good, results are broadly comparable to existing non-invasive markers (145) and their value in longitudinal assessment of disease needs further assessment and validation. Work in this regard is ongoing, for example in the Europe-wide LITMUS study which is focussed on developing and validating new non-invasive biomarkers for NAFLD and NASH (https://litmus-project.eu). Furthermore, whilst some health economic assessments have been attempted (146), more work in this area is required.

1.12 Principles of NAFLD Management

1.12.1 Adopting a multidisciplinary approach

Much of the initial management of NAFLD is able to take place in primary care, provided adequate support structures are in place, which is important given the prevalence and high disease burden. A multidisciplinary team (MDT) approach involving clinicians and allied health professionals is recommended taking into account both liver and cardio-metabolic

morbidity and mortality associated with NAFLD (104,147,148). This will target weight management and other cardiovascular risk factors such as smoking and exercise. Nevertheless, higher quality and more detailed data to evaluate both the outcomes and the cost-effectiveness of the MDT approach is required.

1.12.2 Dietary and lifestyle measures

Weight reduction achieved through dietary and lifestyle measures remains the current gold standard management for the treatment of NAFLD (149,150). 5-10 % weight loss is known to improve both diabetes and cardio-metabolic health in general (151). Furthermore a subgroup analysis of the recent DiRECT study investigating low calorie diets for the treatment of diabetes, which involved participants following a meal replacement plan of approximately 825 calories for 12 weeks (152,153), resulted in a significant reduction in liver fat of around 12-13 %, with corresponding weight loss of between 13-16 kg, and importantly, this reduced liver fat to non-NAFLD levels (<5 %) (154).

With regards to diet, studies indicate that patients who consume a Mediterranean type diet are likely to derive benefit in reduced liver inflammation (ALT) (155), lower liver fat and improved insulin handling (156,157), and current joint European and American guidance recommends adoption of this type of diet in patients with NAFLD (104). Nevertheless, most emphasis should be given on calorie reduction which will be most practically achieved through reduced portion sizes and review of the types of food consumed.

Patients can be supported to make dietary changes with the aid of dietitian. Additionally, signposting or referral to community based weight and exercise programmes, including for example commercial programmes such as Slimming World or Weight Watchers (WW), may be useful for some patients (151).

Referrals to other lifestyle intervention programmes, for example smoking cessation services, which are proven to be cost-effective, will also be useful for patients (158).

Exercise interventions have also been shown to be associated with improvement in NAFLD. Those with higher physical activity levels appear to be have lower prevalence of NAFLD irrespective of weight (159) and increasing physical activity in those with NAFLD modestly reduces liver fat, again irrespective of weight loss (160).

Nevertheless, as is well known, diet and lifestyle modifications used to achieve weight loss are difficult to maintain longer term. As such, novel management strategies, interventions and medical therapies are required to assist the weight reduction process, as well as to target the disease processes and attendant complications associated with NAFLD.

1.13 Emerging therapeutic options for NAFLD

1.13.1 Weight loss surgery

Surgical procedures for weight-loss in obese patients are known to be a highly effective with patients able to lose between 10-25 % of their original body weight depending on the type of procedure performed (161). Weight loss surgery leads to the resolution or improvement of obesity and its complications such as T2DM, dyslipidaemia, hypertension, and obstructive sleep apnoea (162). Multiple studies have also shown improvement in NAFLD in those undergoing such surgery (163). Nevertheless, work remains to establish precisely which procedure is best and the mechanisms underlying the improvements observed as not all patients benefited in studies that have thus far been undertaken (163). Current European and American guidance acknowledges that there may be a role for weight loss surgery in NAFLD (164) though there currently is no explicit UK NICE guidance in this area beyond previous guidance for those with diabetes and obesity.

1.13.2 Pharmacological therapies

There are as yet no directly licenced pharmacotherapies specifically for NAFLD or NASH. Nevertheless, some therapies are licenced or available for other illness which may also have a positive impact on NAFLD or NASH. Current UK NICE guidance reiterates lifestyle interventions in line with obesity management for the treatment of NAFLD and suggests consideration of 2 therapies - pioglitazone, and vitamin E – which have been shown to have some benefit in those with NASH and or advanced fibrosis (149,165). Despite controversies regarding its possible adverse effects, pioglitazone, a thiazolidinedione which reduces insulin resistance by activating the peroxisome proliferator-activated receptor gamma (PPAR-G) may also help to reduce cardiovascular risk, and be safer than previously thought (166,167). Vitamin E, an antioxidant, has been demonstrated to have an impact against NASH but not on liver fibrosis and its impact in patients with T2DM may be limited (168). Questions also persist around its long term safety (169).

A number of other agents, including both established medications and more novel experimental therapies are also in development and or undergoing clinical testing (see Table 1.3). Over the next decade it is hoped that some of these may pass through the regulatory hurdles for use in clinical practice specifically for NAFLD.

1.13.2.1 GLP-1 therapy for the treatment of NAFLD

One promising therapy for NAFLD is glucagon-like-peptide-1 (GLP-1) analogue therapy. GLP-1 analogues mimic the incretin actions of the body's own gut hormone GLP-1 which is released from entero-endocrine L-cells in the gut, to augment insulin secretion in response to an oral glucose load. Chapter 4 describes the mechanisms of action of GLP-1 agonists in

further detail and the various effects including on weight and glycaemic control this class of agent has within the body.

GLP-1 agonist therapy is well established in clinical practice as an effective glucose lowering and weight loss intervention and is licenced by NICE in the UK for patients with poorly controlled diabetes. With regards to potential use in NAFLD, studies to date have demonstrated that the GLP-1 agonist Liraglutide caused a dose-dependent decrease in ALT in those with abnormal liver chemistry at baseline (170). Subsequent prospective studies, in particular the seminal LEAN study, demonstrated histological improvement and resolution of NASH with Liraglutide with 36% of patients showing resolution of NASH (compared with 9% in the placebo arm) (171). Other studies using other GLP-1 agonists Exenatide and Lixisenatide have also shown improvements in hepatic insulin sensitivity, liver biochemistry and lipid content (172,173).

To complement these findings, there is now also well-established evidence from large trials that demonstrates reduced cardiovascular risk associated with the GLP-1 agonists Semaglutide and Liraglutide (174,175) which is of high importance considering the high cardio-metabolic burden with NAFLD. As such, this growing body of evidence suggests that GLP-1 therapy should be made more widely available to patients irrespective of diabetes status as they appear significant clinical benefit with a relatively safe side effect profile.

Further work is ongoing to provide more details on the potential utility of GLP-1 therapy in NAFLD including a large phase 2 study in patients with NASH using liver biopsy endpoints. This trial is assessing 3 different doses of Semaglutide *vs.* placebo and is due to complete later in 2019 (176). Long-acting, once weekly, GLP-1 analogue therapy is now also used in clinical practice with data showing that weight and liver chemistry improve with treatment (177,178) and further studies are ongoing (179,180). Finally, more novel compounds with multiple agonist properties are in development. For example, HM1522 is a GLP-1/GIP/glucagon triple agonist and is currently being trialled for use in NAFLD (181).

The mechanisms by which GLP-1 analogues act to improve NAFLD remains to be fully elucidated and there is ongoing debate as to whether the GLP-1 receptor is expressed in human hepatocytes. I*n vitro* studies have suggested that GLP-1 agonism can decrease de novo lipogenesis (DNL) in human hepatocyte primary cultures (182) though this may be achieved via indirect means and other studies have not been able to localise the GLP-1 receptor on hepatocytes (183). Furthermore, the confounding issue of weight loss and improvements in glycaemic control remain to be disentangled from the potential direct benefits of GLP-1 therapy on the liver in NAFLD.

1.13.2.2 Emerging therapies for NAFLD

There are various treatment strategies which may be utilised to target NAFLD. These include agents which work on glucose handling pathways and which reduce insulin resistance and diabetes risk, agents which work to improve lipid handling, and agents which serve to reduce liver inflammation and or liver fibrosis. Though there are no pharmacological agents currently licenced for the specific treatment of NAFLD or NASH, a number of agents under investigation may be available and have licences for other indications for example to treat diabetes and dyslipidaemia. Table 1.3 summarises emerging pharmacological therapies that may have future utility in treating NAFLD as presented in the review by Marjot *et al (2)*.

Table 1.3: Emerging potential pharmacological options for NAFLD

*Table reproduced and adapted from Marjot et al (2). *Agents which have a licence or are available for use to treat other medical conditions.*

Agent	***Mechanism of action***	***Impact on liver biochemistry and / or non-invasive assessments of NAFLD disease status***	***Impact on liver histology***	***Comments***	***References***
Anti-diabetic, glucose lowering agents (with existing license for the treatment of type 2 diabetes)					
Metformin*	Multiple mechanisms including AMPKinase activation	Some but not all studies, have shown improvements in liver chemistry and hepatic steatosis	Meta-analyses have shown no significant benefit on liver histology	Remains the first line pharmacotherapy for patients with T2D. Its use specifically for the treatment of NAFLD is not recommended.	(184,185)
Pioglitazone*	PPARγ agonist	Consistent improvements in liver chemistry and reductions in hepatic steatosis.	Decreased steatosis and inflammation, with resolution of NASH. Some evidence to suggest that fibrosis may improve	Despite the histological benefits, widespread use is currently limited, potentially by concerns over adverse effects including weight gain, bone fracture risk and fluid retention.	(186–188)
Liraglutide*	GLP-1 analogue	Improved liver chemistry and decreased hepatic steatosis.	Decreased steatosis, inflammation and resolution of NASH without worsening of fibrosis	To date, only data from small studies have been published and the relative contribution of weight loss and improvement in glycaemic control to the observed benefits in NASH are yet to be determined.	(171,189)
Exenatide*	GLP-1 analogue	Improvements in liver chemistry and fatty liver index	n/a		(172,190,191)
Lixisenatide*	GLP-1 analogue	Improvement in ALT in obese and overweight individuals	n/a		(173)
Semaglutide*	GLP-1 analogue	n/a	n/a	Phase 2 studies currently recruiting	(176)

Dulaglutide*	Long acting GLP-1 analogue	Improved liver chemistry	Histological report of improvement in a single case	No dedicated histological studies performed to date. Phase 2 studies are recruiting.	(177,178)
HM1522	GLP-1/GIP/ Glucagon triple agonist	n/a	n/a	Early phase 2 studies currently recruiting.	(181)
Sitagliptin*	DPPIV inhibitor	Inconsistent results and some studies have failed to show improvements in liver chemistry or liver TAG content.	Limited histological evidence of benefit with inconsistent results; improvement in NAS in some studies but not all.	Overall the data do not suggest that there is significant benefit when used as a treatment for NAFLD.	(192,193)
Empagliflozin*	SGLT2 inhibitor	Improvements in liver chemistry and decreased hepatic TAG content	Some evidence for histological improvement in an open label study.		(194–196)
Canagliflozin*	SGLT2 inhibitor	Some evidence of improvements in liver chemistry and surrogate markers of fibrosis in very small numbers of patients. Reduction in liver TAG content and increased hepatic insulin sensitivity	Improvement in inflammation and steatosis in a single biopsy study.	Uncontrolled liver biopsy study, with very small numbers (n=9) with no placebo or other comparator arm	(197)(198)
Ipragliflozin*	SGLT2 inhibitor	Some evidence for improvement in liver chemistry with variable changes in markers of fibrosis.	Single case report of histological improvement in steatosis, inflammation and ballooning		(199–201)
Dapagliflozin*	SGLT2 inhibitor	Improvements in liver chemistry and reduction in liver TAG content. No impact on increased hepatic insulin sensitivity	n/a		(202,203)
Licogliflozin	Dual SGLT1 and 2 inhibitor	n/a	n/a	Early phase 2 studies currently recruiting.	(204)

Lipid lowering therapies					
Simvastatin*	HMG CoA reductase inhibitor	Some evidence for improved liver chemistry	No evidence of benefit in a small (n=16) study	Study may have been underpowered to detect histological improvement.	(205,206)
Atorvastatin*	HMG CoA reductase inhibitor	Some evidence for improved liver chemistry	No improvement on inflammation or fibrosis on liver histology		(207–209)
Pitavastatin*	HMG CoA reductase inhibitor	Some evidence for improved liver chemistry	No improvement on liver histology		(210,211)
Rosuvastatin*	HMG CoA reductase inhibitor	Some evidence for improved liver chemistry	Resolution of NASH in 19/20 patients	Histological data are from an uncontrolled study	(212)
Omega3-fatty acids*		Some evidence for improved liver chemistry and decreased steatosis	Little evidence to suggest significant improvements in inflammation or fibrosis		(213)
Fenofibrate*	PPARα agonist	Improved liver chemistry and steatosis on ultrasound scanning	n/a		(214,215)
Pemafibrate*	PPARα agonist	n/a	n/a	Phase 2 studies are currently actively recruiting.	(216)
Saroglitazar*	Dual PPARα/ γ agonist	n/a	n/a	Phase 2 studies are currently actively recruiting	(217)
Evolocumab*	PCSK9 inhibition	n/a	n/a	Safe and well tolerated. No deterioration in liver chemistry.	(218)

				Dedicated studies in NAFLD are yet to be performed.	
Liver-targeted therapies (anti-inflammatory and anti-fibrotic)					
Obeticholic acid*	FXR agonist	Improvements in liver chemistry	Resolution of NASH and improvements in fibrosis	Phase 3 studies are currently recruiting (including patients with NASH cirrhosis).	(219)
GS-9674	FXR agonist	n/a	n/a	Biochemical improvements in patients with PSC, dedicated studies in patients with NAFLD are currently recruiting.	(220)
Tropifexor (LJN452)	Non-bile acid FXR agonist	n/a	n/a	Phase 2 studies currently recruiting	(221)
Elafibranor	Dual PPAR α/δ agonist	Improvements in liver chemistry as well as lipid profiles and glucose homeostasis in patients with T2D.	Resolution of NASH without worsening in fibrosis	Phase 3 studies are currently recruiting	(222)
Lanifibranor (IVA337)	Pan-PPAR agonist	n/a	n/a	Phase 2 studies are currently actively recruiting	(223)
Cenicriviroc	Dual CCR2/5 antagonist	No changes in liver chemistry or steatosis	Primary end point for resolution of NASH was not met in the phase 2 studies, but improvements in fibrosis	Some improvement in systemic markers of inflammation. A large phase 3 is currently recruiting	(224)

			were observed.		
Vitamin E*	Multiple mechanisms including anti-oxidant actions	Inconsistent improvements in liver chemistry and steatosis	Resolution of NASH in some studies, but not all; no impact on fibrosis		(187,225)
GR-MD-02	Glactin-3 protein inhibition	No changes in liver chemistry in the early phase studies published to date.	No significant change in NAS, although ballooning decreased. No change in fibrosis	Liver biopsy studies performed in patients with NASH cirrhosis.	(226,227)
NGM282	FGF 19 analogue	Improvements in liver chemistry and steatosis	Early phase 2 study has demonstrated improvements in steatosis, inflammation and fibrosis	A larger phase 2 study is actively recruiting.	(228,229)
LY2405319	FGF 21 analogue	n/a	n/a	Evidence for lipid lowering in patients with type 2 diabetes	(230)
Pegbelfermin (BMS-986036)	FGF 21 analogue	Decreased hepatic steatosis	n/a	A series of phase 2 studies are currently recruiting.	(231)
Resmetirom, MGL-3196	Thyroid Hormone Receptor-β agonist	Decreased hepatic steatosis	Resolution of NASH and decreased steatosis	A phase 3 study is actively recruiting	(232,233)
Selonsertib	Apoptosis Signal-Regulating Kinase 1 Inhibitor	Improvements in liver chemistry.	Improved fibrosis at the highest doses.		(234)
Simtuzumab	lysl oxidase-like-2 inhibition	No significant changes in liver biochemistry.	No improvement in NAS or fibrosis.	No benefit on histological analysis or on clinical outcomes	(235)

*Pentoxifylline**	Multiple mechanisms including anti-inflammatory actions and reduction in free oxygen radial generation	No change in liver chemistry	Evidence for improvement in NAS and fibrosis		(236,237)
Emricasan	Pan-caspase inhibition	Improved liver chemistry	n/a	Short duration early phase studies only have been reported	(238,239)
SGM-1019	Inflammasome inhibition	n/a	n/a	Safe and well tolerated in a phase 1 study. A phase 2 study is actively recruiting	(240,241)
JKB-121	Toll-like receptor 4 antagonism	No impact on hepatic steatosis	n/a	Relatively high adverse event rate leading to drug withdrawal	(242)
IMM-124E	Limitation of endotoxin exposure	n/a	n/a	Phase 2 study completed, but results not yet reported	(243)
Amlexanox*	IKKb and TANK-binding kinase 1 inhibition	Some evidence for decreased hepatic steatosis in patients with diabetes	n/a		(244)
Tipelukast (MN-001)	Phosphodiesterase and 5-lipoxygenase inhibition and leukotriene receptor antagonism	n/a	n/a	Phase 2 studies are currently recruiting	(245)

DS102	5-lipoxygenase inhibition	n/a	n/a	Phase 2 studies are currently recruiting	(246,247)
BI 1467335	Vascular adhesion protein-1 inhibition	n/a	n/a	Early phase 2 studies are currently recruiting	(248)
MSDC-0602K	Mitochondrial pyruvate carrier inhibition	Evidence for improved metabolic as well as surrogates of hepatic inflammation and fibrosis on interim analysis	n/a	Large phase 2 study (EMMINENCE) currently recruiting	(249)(250)
Hepatic lipid metabolism					
GS-0976	Acetyl-Coenzyme A Carboxylase Inhibition	Improved liver chemistry and hepatic steatosis	n/a		(251,252)
Aramchol	Stearoyl coenzyme A desaturase 1 inhibition	No change in liver chemistry, but decreased hepatic steatosis	n/a	Phase 2b completed although not reported.	(253)
Gut microbiome					
Probiotics*	Alteration in gut microbiome	Improvements in liver chemistry and steatosis	n/a		(254)
Solithromycin	Macrolide antibiotic	n/a	Improved NAS in small proof-of-concept study		(255)
Faecal microbiome transplantation		n/a	n/a	Several phase 2 studies currently recruiting	(256–258)

1.14 Summary

This DPhil thesis will outline novel research undertaken on the topic of non-alcoholic fatty liver disease. In particular, it will review and analyse outcomes arising from the current management of NAFLD in one particular service in Oxfordshire UK (Chapter 2). It will then go on to describe novel urinary steroid based non-invasive biomarkers to diagnose and stage NAFLD (Chapter 3). Finally, it will outline research investigating GLP-1 agonists as novel treatments agents for NAFLD (Chapter 4).

Chapter 2 A multidisciplinary approach to the management of NAFLD: biomarkers, interventions and efficacy

The research contained in this chapter has been published in the academic medical journal Frontline Gastroenterology in a research paper titled ‘A multidisciplinary approach to the management of NAFLD is associated with improvement in markers of liver and cardio-metabolic health’ (259) on which I am the first author. The research presented was initiated and led by myself as first author with contributions from co-authors. The text contained in this chapter is adapted from the published research paper. It was pleasing to find that it was further accompanied by a commentary piece in the same journal which reviewed our published manuscript (148).

2.1 Introduction

As outlined in Chapter 1, NAFLD is a globally prevalent public health burden affecting up to 30% of many populations. With this epidemic continuing to grow alongside the global obesity and type 2 diabetes (T2DM) pandemics (260), novel clinical approaches and interventions are required and are being trialled to try to reduce the disease burden. Whilst the health service awaits the first licenced medical therapies to treat NAFLD, healthcare providers have begun to develop services based around existing therapies and interventions that are of use in treating NAFLD. As these emerging services are being established, there is a need to evaluate their value, including both clinical effectiveness – as measured via health outcomes – and their cost-effectiveness, to help inform, improve, and better target future services at both patient and population healthcare levels.

Traditionally, NAFLD has been managed by hepatologists with the knowledge that advanced fibrosis (stages F3-F4 which includes liver cirrhosis) confers a significantly increased risk of hepatic decompensation and development of hepatocellular carcinoma (HCC) as well as being associated with reduced transplant-free survival, increased liver-related and all-cause mortality (3,37,261). However, informed by more recent data over the past 15 years demonstrating that the principle cause of morbidity and mortality from NAFLD is that from

cardiovascular and metabolic disease (3,37), there is increasing appreciation amongst healthcare professionals that a multidisciplinary approach to its management is necessary (148).

With up to 70% of patients with T2DM estimated to suffer from NAFLD, T2DM being the strongest risk factor for NAFLD disease progression (29) and those with both NAFLD and T2DM having accelerated liver disease progression and more prevalent and severe complications of diabetes (262,263), diabetes specialists and metabolic physicians are taking a keener interest in NAFLD and in the UK are beginning to join and advise hepatologists in managing such patients, with joint services established for example at Imperial College Healthcare NHS Trust and Oxford University Healthcare NHS Trust (OUH). In these clinics, management focuses on modification of risk factors for NAFLD progression and cardiovascular disease within a multidisciplinary framework (33,147,264) and, as such, there is an added recognition and need for patients to have support from allied health professionals including dietary and lifestyle experts.

2.1.1 Current intervention and management for NAFLD through a multidisciplinary metabolic hepatology clinic approach

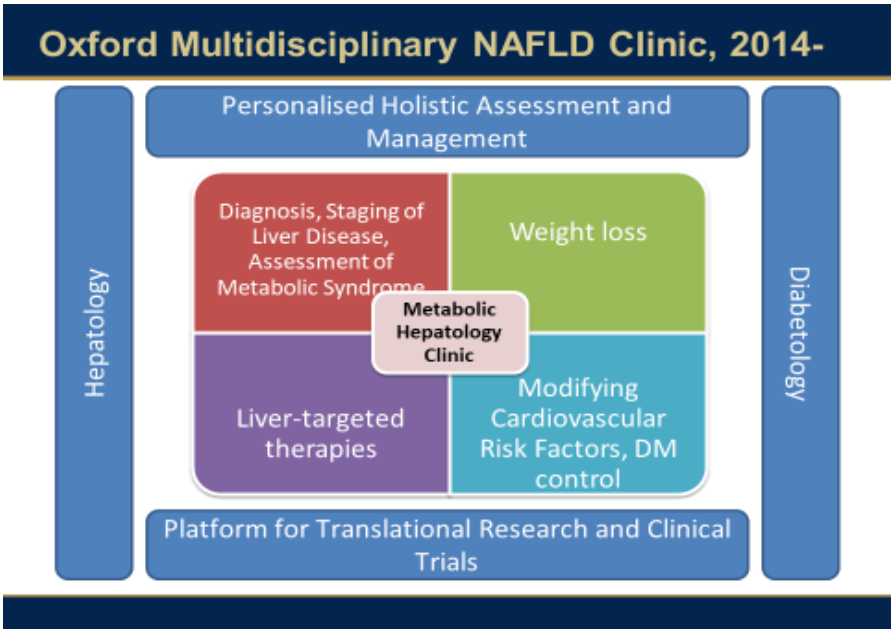
The Oxford University Hospitals NHS Foundation Trust (OUH) delivers a weekly secondary / tertiary multidisciplinary Metabolic Hepatology clinic, managing patients from across Oxfordshire, UK, and the surrounding regions. Referrals to the clinic are received from both primary care and secondary care settings. OUH works closely with the Oxfordshire Clinical Commissioning Group and has produced guidelines (265) to assist primary care professionals to investigate and refer of patients. These guidelines assist GPs to risk stratify patients and help ensure that those at high risk of advanced liver disease are referred to and managed in secondary care (Note: this pathway was updated in November 2017 after the

period covered by the analysis contained in this chapter. Appendix 7.1 details the pathway both pre the 2017 update and the updated 2017 version).

From January 2015 to November 2017, local guidance recommended that patients with suspected NAFLD in primary care first underwent risk-stratification with the NAFLD fibrosis score (NFS) with referral of those patients with indeterminate or high risk scores to the Metabolic Hepatology clinic. However, referrals without risk stratification were not refused and risk stratification was performed in clinic thereafter. If the diagnosis was unclear, patients were referred to the General Hepatology clinic at OUH. In clinic, further risk stratification is undertaken through tools including the NAFLD Fibrosis Score, Fib-4 score and/or transient elastography (FibroScan), and subsequent follow-up appointments scheduled in the Metabolic Hepatology clinic where appropriate.

The clinic is jointly led by hepatologists and diabetologists / metabolic physicians. The aim of the clinic is to improve liver-related and cardiovascular health in patients with NAFLD/NASH. Accordingly, appropriate disease staging at presentation aims to identify those at high risk of adverse outcomes who would benefit from more intensive management. A range of lifestyle and medical interventions are employed with emphasis given to weight management and the achievement of meaningful weight loss in those who are overweight or obese via dietary and lifestyle modifications. Medications are used to aid reduction of cardiovascular risk, for example, the use of antihypertensive and statin therapy, as well as to improve diabetes control where required. For the control of diabetes, therapies that are weight-neutral or encourage weight loss such as SGLT-2 inhibitors and GLP-1 agonists are favoured (171,195,266,267). These newer diabetes therapies are further beneficial as they also reduce cardiovascular risk (268–270).

Figure 2.2. The Oxford Metabolic Hepatology Clinic. Figure courtesy of Dr Jeremy Cobbold.



The clinic is supported by specialist nurses performing Fibroscan and anthropometrics immediately prior to the medical consultation and by specialist practitioners via the *Here for Health* service. This is a special service at OUH that bridges the link between the acute hospital setting and currently available community services. It provides a range of health and wellbeing advice including weight management (diet, lifestyle and exercise), but also smoking cessation, alcohol reduction and signposting to mental health services (www.ouh.nhs.uk/patient-guide/here-for-health/default.aspx).

Blood testing, imaging, liver biopsy and screening for hepatocellular carcinoma in patients with established cirrhosis are performed where clinically appropriate and in accordance with relevant clinical guidelines.

Follow-up for patients in the clinic is based on patient need. If a patient undergoes risk stratification and is found to have mild disease (low-risk NAFLD Fibrosis Score or Fib-4,

FibroScan <8 kPa without adjustable metabolic complications, or liver biopsy F≤2 in the absence of metabolic complications), and can be managed well in primary care, they are typically discharged with advice to repeat community-based risk stratification at 2-3 years to assess for disease progression and re-referral if required. Exceptions include those with complex metabolic comorbidities, florid NASH in young people and others at the clinician's discretion. Whilst ideally all patients with liver fibrosis of any stage would be followed up in secondary care as they are at risk of developing complications, the high burden of NAFLD means that this is not possible for all patients, and a pragmatic approach in collaboration with primary care colleagues is necessitated.

Those patients undergoing diagnostic procedures such as liver biopsy or significant therapeutic intervention (lifestyle or medical) are typically offered follow-up at 3-6 months depending on individual patient need. For those with stable advanced fibrotic liver disease (F3) but who are not cirrhotic, annual follow-up is arranged. For those with compensated NASH cirrhosis, follow-up is routinely at six monthly intervals.

All patients are seen by a specialist clinic nurse (for anthropometry and Fibroscan if required) and a hepatologist at each appointment, with a diabetologist seeing most patients with diabetes and all patients with poorly controlled diabetes. All patients are offered a *Here for Health* review at their initial visit, and if further issues arise, at subsequent visits. Additionally, *Here for Health* offers an open-access walk-in service if patients wish to attend at an alternative time.

2.1.2 Aims and hypothesis

This study aimed to evaluate the impact of a metabolic hepatology clinic that adopts a multidisciplinary, holistic approach to treat NAFLD and used a number of markers of liver

and cardio-metabolic health to assess its performance. This includes thorough assessment of surrogate markers of liver injury, diabetes control and cardiovascular risk, and how these changed with management. Furthermore, this study evaluated the effects of the clinic on quality adjusted life expectancy (QALE), and estimated the cost-effectiveness of the approach.

The hypothesis is that the multidisciplinary metabolic hepatology clinic is both clinically and cost effective in managing patients with NAFLD.

2.2 Methods

2.2.1 OUH metabolic hepatology clinic study analysis

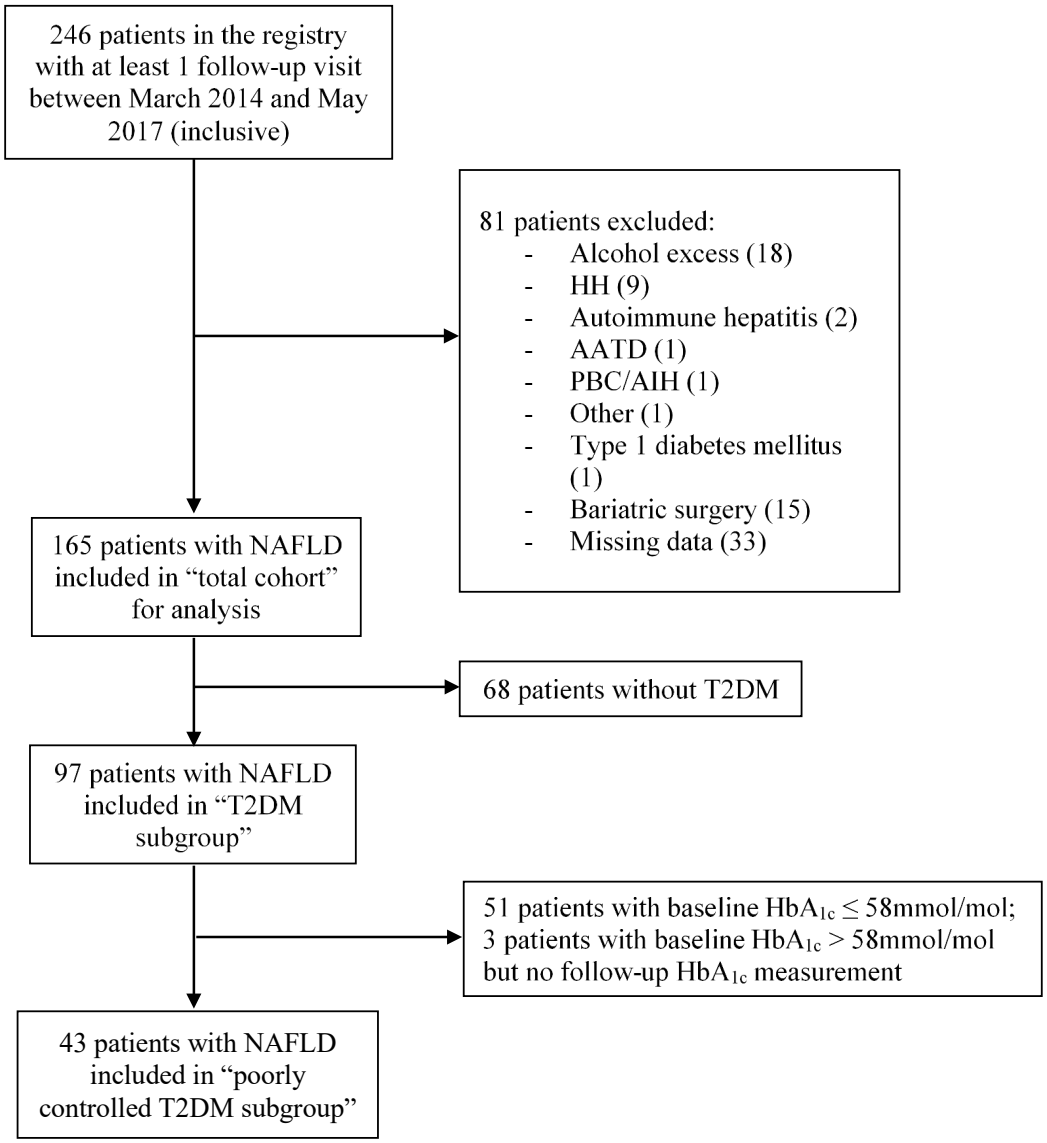
A retrospective analysis of all patients who were managed through the metabolic hepatology clinic at OUH between its inception in March 2014 until May 2017 was performed. Patients were included in the analysis if they had (i) attended the clinic at least twice, (ii) had an alanine aminotransferase (ALT) level recorded at baseline and at their latest (most recent) clinic visit, and (iii) had weight recorded at baseline. Clinical data from all patients attending since clinic inception was collected on a clinic proforma in real time at each clinic visit and subsequently recorded on a centrally-held, secure, departmental clinical spreadsheet for audit purposes.

Diagnosis of NAFLD was made according to UK National Institute for Health and Care Excellence (NICE) and related guidance (149); either radiologically (liver ultrasound or liver magnetic resonance imaging), histologically on liver biopsy, or on the basis of persistently elevated liver enzymes (elevated ALT and/or AST levels (> 40 IU/L) in the context of features of the metabolic syndrome and where other causes of liver pathology had been excluded. Patients with known hepatic co-morbidity, patients who did not have a diagnosis of

NAFLD, those who had type 1 diabetes and those who had previously undergone or who underwent bariatric surgery during the follow-up period were excluded from the analysis (Figure 3.2).

Figure 2.3: Selection of cohort for analysis from the metabolic hepatology clinic

Flow chart of total cohort and selection from the full clinic registry and subgroup selection used to perform analyses



Abbreviations and definitions: Alcohol excess defined as an average intake of > 21 (male) or 14 (female) units per week for a prolonged period within 5 years prior to baseline visit; HH, hereditary haemochromotosis (HFE C282Y homozygous and C282Y/H63D compound heterozygous); AATD, alpha-1 antitrypsin deficiency (ZZ and SZ genotype); PBC/AIH, primary biliary cirrhosis-autoimmune hepatitis overlap syndrome; T2DM, type 2 diabetes mellitus; HbA_{1c}, glycated haemoglobin.

Patients underwent assessments of liver and cardio-metabolic health using current routine non-invasive tools including measurement of serum ALT, AST and liver stiffness (FibroScan), a validated surrogate marker of liver fibrosis (271). Calculation of the Fib-4

score (272) provided additional non-invasive assessment of risk of advanced liver fibrosis. Liver biopsy, still the reference standard for diagnosis and assessment of disease severity was performed where clinically indicated.

Cardio-metabolic assessment included measurement of weight, body mass index (BMI), alcohol intake, blood pressure, lipids and diabetes control (HbA1c). All patients were asked to complete a qualitative 7-day food diary in advance of clinic attendance. Cardio-metabolic risk factors were defined using standard measures employed in clinical practice: hypertension if blood pressure was ≥140/90 mmHg, or if the patient was taking antihypertensive medication; type 2 diabetes according to WHO criteria (HbA1c ≥48 mmol/mol), if the diagnosis was already established at baseline or if patients were taking anti-diabetic medication; dyslipidaemia according to local thresholds (triglycerides >1.7 mmol/L and/or high-density lipoprotein (HDL) cholesterol <1.0 mmol/L (male), <1.3 mmol/L (female) and obesity if BMI was ≥30 kg/m^2. All patients had 10-year cardiovascular risk assessment (absolute and relative risk) calculated retrospectively using the QRISK3-2017 score (273). The QRISK3 prediction algorithm is based on routine data collected for over 10 million patients from 1309 UK primary care general practices using separate derivation and validation cohorts to ensure good performance (274). The algorithm “calculates a person's risk of having a heart attack or stroke with the next 10 years and presents the average risk of people with the same risk factors as those entered for that person” (273). Each patient’s current drug history was recorded at each clinic visit. For patients with T2DM, data on prescribed medical therapies was collated from clinic letters and cross-referenced by checking written clinic proformas.

The primary outcome was change in ALT level between baseline and latest (most recent) clinic visit. Secondary outcomes were changes in weight, glycated haemoglobin (HbA1c), aspartate aminotransferase (AST), lipid profile (total cholesterol, HDL, triglycerides), systolic blood pressure, transient elastography, Fib-4 score, NAFLD fibrosis score (NFS), and QRISK3 cardiovascular risk score. Patients with a missing variable value at baseline and/or

latest clinic visit were not included for paired analysis of that variable. A subgroup analysis was also performed on patients with (i) T2DM at baseline (baseline HbA1c >48 mmol/mol), and (ii) poorly controlled T2DM at baseline, (baseline HbA1c >58 mmol/mol), who also had a latest clinic HbA1c measurement.

2.2.2 Economic analysis using the UKPDS Outcomes Model 2 to determine cost effectiveness

The UK Prospective Diabetes Study Outcomes Model (version 2.0, UKPDS-OM2), a lifetime simulation model for patients with T2DM which has been extensively validated, was used to model and predict changes in quality adjusted life expectancy (QALE) as well as the potential cost-effectiveness of our approach (275). The UKPDS-OM2 was firstly applied to all patients with T2DM at baseline (HbA1c >48 mmol/mol) and subsequently in patients with poorly controlled T2DM at baseline (HbA1c >58 mmol/mol). The model, incorporates phenotypic data and changes to various modifiable cardio-metabolic risk factors over time. A standard, accepted modelling approach was adopted to compare outcomes in the changes observed in those attending the clinic to outcomes in the absence of these changes. The design of the model and analysis adhered to standard good practice guidelines, such as the Consolidated Health Economic Evaluation Reporting Standards (CHEERS) checklist (http://www.ispor.org/TaskForces/EconomicPubGuidelines.asp) which has been widely adopted, though as this was not a stand-alone economic evaluation but an additional analysis within the wider paper, not every point could be followed.

The UKPDS Outcome Model incorporates changes to various modifiable and non-modifiable cardio-metabolic risk factors. The effects of changes to these parameters in patients with T2DM attending the clinic (intervention group) was measured and compared to a reference group where baseline modifiable risk factors for each patient attending our clinic were held constant (reference group) during the running of the UKPDS model (70 years or death). For the intervention group, the modifiable risk factors of each patient were held constant from

the point of latest follow-up to death or year 70 of the model. Missing baseline data for patients were populated using the mean baseline value of each group of interest. Missing data at subsequent years for the group of interest were populated with the patients' own values from the previous year carried forward.

Costs were based on 2016-17 price levels. 1 year of therapy based on the mean and median follow-up period of 1 year was assumed and costed at £591 based on 1 new consultation (Spec Code 306, Hepatology, POD OPFAMPCL: £285) and 2 follow-up consultations (Spec Code 306, Hepatology, POD OPFUPMPCL £153 each), as per 2016-17 NHS National Tariffs reimbursed to OUH. Complication costs were based on default values incorporated in the UKPDS Outcomes Model version 2.

The UKPDS Outcomes model analysis was designed by Dr Ahmad Moolla, Mr Kenzo Mothashi and Prof Alastair Gray and run by Mr Kenzo Mothashi. Each model scenario was run 5 times using 20,000 loops (averaging across repeated simulations to minimise monte carlo variation) and 250 bootstraps (to derive 95% confidence intervals reflecting model parameter uncertainty). Mean differences between the two groups in life expectancy, quality adjusted life expectancy and costs were calculated, and an incremental cost-effectiveness ratio (difference in costs/difference in QALE) was calculated and compared with the current NICE position, where interventions with a cost-effectiveness ratio of less than £20,000 per quality-adjusted life year (QALY) are considered good value for money. When calculating cost-effectiveness, all future costs and outcomes were discounted at an annual rate of 3.5% in line with current UK guidelines.

2.2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7.0). Data were non-parametrically distributed as assessed by the D'Agostino-Pearson omnibus test. Continuous variables are quoted as median (range), and categorical variables as numbers and percentages. Non-parametric tests were used to assess for statistical significance (Wilcoxon signed rank test for paired baseline and latest visits, Mann-Whitney U and Chi-square (χ^2) tests for comparing between the T2DM and non-T2DM subgroups) and the threshold for significance was set at the 5%. Endpoints were set as baseline to latest follow-up and response.

2.3 Results

2.3.1 Baseline Characteristics

165 patients with NAFLD were followed from baseline until their latest clinic visit. Baseline characteristics are described in Table 2.1. Median interval between baseline and latest follow-up clinic visit was 13.3 months (range 2–34), with a median of 2 follow-up visits (range 1-5).

Patients reported taking a median of 4 prescription medications (range 0-17) at baseline. Oral hypoglycaemic agents were the most commonly prescribed medication with 52 % taking ≥1 agent, followed by anti-hypertensive therapy (51 %) and statin therapy (42 %).

Table 2.4 Baseline characteristics of the metabolic hepatology clinic cohort and T2DM and non-T2DM subgroups

Baseline characteristic, median (range) or number (%)	**Total cohort** **n = 165**	**T2DM subgroup** **n = 97**	**Non-T2DM subgroup** **n = 68**	**#p-value**
Demographic				
Age, years	53 (16-79)	57 (29-79)	47.5 (16-69)	<0.0001
Sex, male	106 (64.2%)	62 (63.9%)	44 (64.7%)	0.92
Caucasian ethnicity	132 (80.0%)	78 (80.4%)	54 (79.4%)	0.87
Metabolic				
‡ HbA_{1c}, mmol/mol	46 (25-124)	59 (35-124)	36.5 (25-46)	<0.0001
‖ Total cholesterol, mmol/L	4.4 (2.4-9.0)	4.0 (2.4-7.7)	4.9 (2.7-9.0)	0.001
‖ HDL, mmol/L	1.0 (0.5-2.4)	1.0 (0.5-1.7)	1.0 (0.8-2.4)	0.24
◊ Triglyceride, mmol/L	1.93 (0.52-17.01)	2.13 (0.52-10.71)	1.72 (0.68-17.01)	0.10
§ Systolic blood pressure, mmHg	140 (105-190)	140 (105-189)	138 (108-190)	0.45
Metabolic syndrome (MetS)				
BMI, kg/m^2	33 (23.9-72.1)	34 (23.9-52.6)	33 (24.2-72.1)	0.48
Obesity (BMI>30kg/m^2)	120 (72.7%)	71 (73.2%)	49 (72.1%)	0.87
Hypertension	93 (56.4%)	72 (74.2%)	21 (30.9%)	<0.0001
Dyslipidaemia	105 (63.6%)	71 (73.2%)	34 (50.0%)	0.002
Number of MetS components, /4	3 (0-4)	3 (1-4)	2 (0-3)	
Lifestyle				
Alcohol, units/week	0 (0-21)	0 (0-21)	0 (0-20)	
Current Smoker	10 (6.1%)	6 (6.2%)	4 (5.9%)	0.94
Ex-smoker	53 (32.1%)	35 (36.1%)	18 (26.5%)	0.19
Liver				
ALT, IU/L	52 (12-215)	50 (12-200)	54 (12-215)	0.16
Abnormal ALT (> 40 IU/L)	111 (67.3%)	60 (61.9%)	51 (75.0%)	0.08
↕ Transient elastrography, kPa	9.2 (3.5-75.0)	10.1 (4.3-75.0)	8.2 (3.5-53.3)	0.01
Biopsy characteristics				
Number (%)	87 (52.7%)	54 (55.7%)	33 (48.5%)	
*NAS, /8	5 (2-8)	5 (2-8)	5 (2-7)	0.75
*NASH	57 (72.1%)	36 (73.5%)	21 (70.0%)	
**Fibrosis stage, (F0-F4)	2 (0-4)	3 (0-4)	2 (1-4)	0.19
F0, (n, %)	1 (1.2%)	1 (1.9%)	0 (0.0%)	
F1 (n, %)	15 (17.2%)	6 (11.1%)	9 (27.3%)	
F2 (n, %)	34 (39.1%)	20 (37.0%)	14 (42.4%)	
F3 (Bridging fibrosis) (n, %)	21 (24.1%)	14 (25.9%)	7 (21.2%)	
F4 (Cirrhosis) (n, %)	16 (18.4%)	13 (24.1%)	3 (9.1%)	

‡ number of patients with baseline measurement, n=136, 88, 48 (of total cohort, T2DM subgroup, non-T2DM subgroup, respectively); ‖ n=113, 68, 45; ◊ n=100, 59, 41; § n=137, 82, 55; ↕ n=133, 73, 60.
#p-value compares T2DM group to non-T2DM group
Biopsy Characteristics provided for available data * NAS (NAFLD activity score) (/8); NASH defined as NAS ≥ 5; Number of patients with measurement per group (percentage with NASH): n=79, 49, 30 in each column group. ** Fibrosis stage (Brunt's scale) (F0-F4); Cirrhosis defined as fibrosis stage F4 or historical diagnosis in the case of 1 missing biopsy score. Number of patients with measurement per group (percentage): n=86, 53, 33.

87 patients (53 %) had a liver biopsy prior to or during the follow-up period (Table 2.1). There was no significant relationship between T2DM status and histological NAFLD activity scores (NAS) (p=0.75). Median NAS in patients with T2DM and without T2DM were both 5. There was also no significant relationship between T2DM status and fibrosis stage (p=0.19) although the majority of patients with biopsy-proven cirrhosis in the cohort had T2DM (13/16, 81 %).

There was no difference in smoking status (past, present or ever smoked) between those with T2DM and those without, though those with T2DM were much more likely to have hypertension (p<0.0001) and dyslipidaemia (p=0.002).

2.3.2 Changes in liver and cardio-metabolic health in all patients

At latest follow-up, median ALT had reduced significantly by 11 IU/L and median AST reduced significantly by 7 IU/L (Table 2.2). Median liver transient elastography also reduced significantly by 1.3 kPa. For Fib-4 and NFS, there were no changes in the proportion of patients switching between categories (risk of presence or absence of advanced liver fibrosis or indeterminate score) (Fib-4: p=0.8, NFS: p=0.7).

Analysis of weight changes revealed that the majority of patients lost weight. The distribution of weight loss is shown in Figure 2.3. Figure 2.3 also summarises changes in cardio-metabolic and liver parameters stratified by weight loss. In this retrospective analysis of clinical data, even modest weight loss of <5 % was associated with improvement in these parameters and suggested that the magnitude of improvement was greater with increased weight loss.

QRISK3 10-year cardiovascular relative risk reduced significantly by 0.1 (5 %). There was no significant change in QRISK3 10-year absolute risk from baseline to latest visit, though when

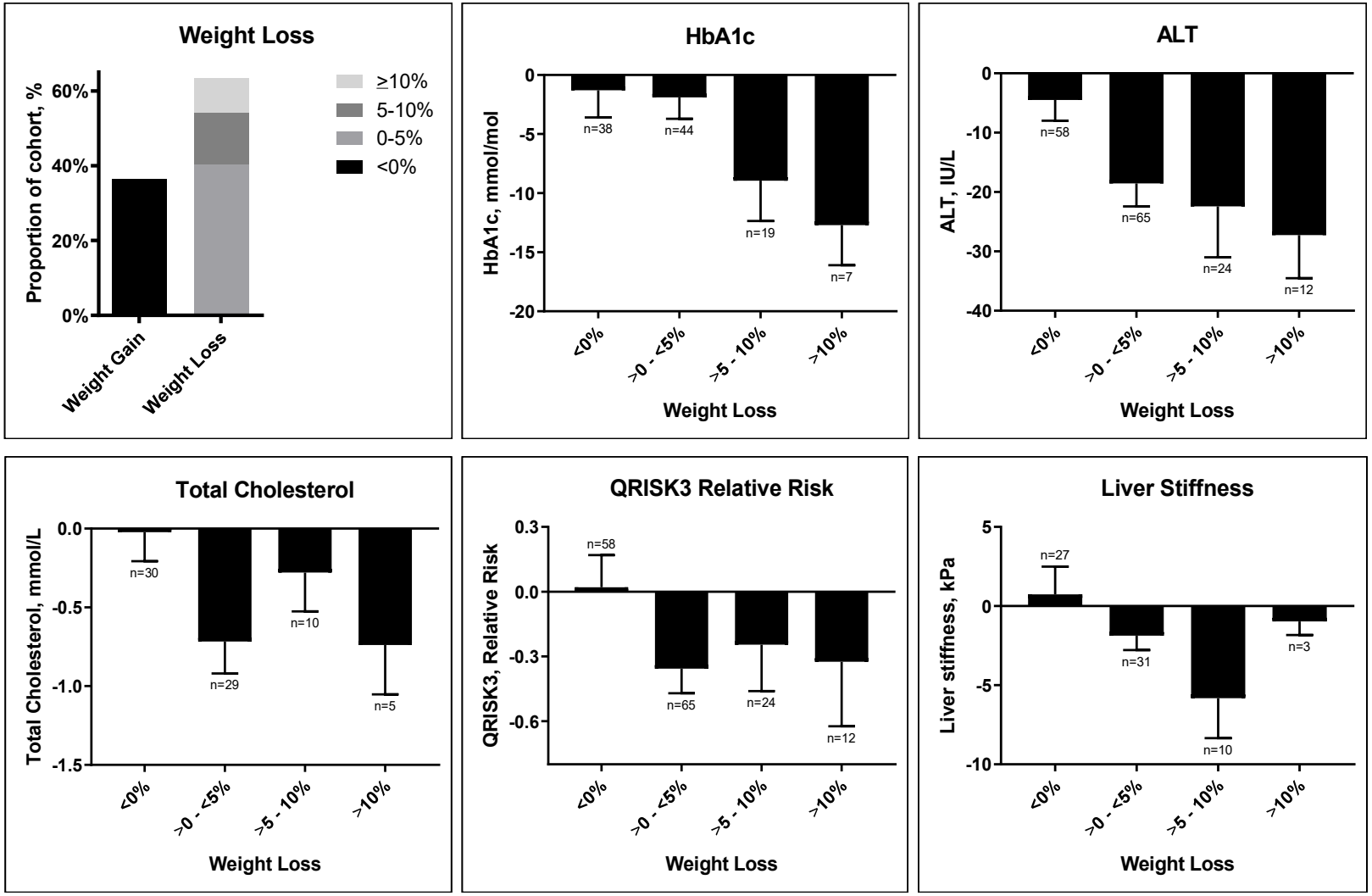
follow-up age, a component of this algorithm, was corrected to ‘age-match’ (remain the same) to baseline, this reduced significantly by 0.8 (6%).

Table 2.5: Change to liver and cardio-metabolic health markers: baseline to latest clinic visit

All values shown are median (range)	Total cohort				T2DM subgroup				Non-T2DM subgroup			
	Base.	Latest	Δ	*P* value	Base.	Latest	Δ	*P* value	Base.	Latest	Δ	*P* value
Liver function test												
ALT, IU/L	52 (12-215)	41 (11-240)	-11	**<0.0001**	50 (12-200)	40 (11-125)	-10	**<0.0001**	54 (12-215)	47 (12-240)	-7	**0.0007**
AST, IU/L	40 (15-171)	33 (14-105)	-7	**0.011**	35 (16-171)	31 (14-63)	-4	0.13	43 (15-116)	38 (15-105)	-5	0.054
Weight												
Weight, kg	97.3 (55.0-206.0)	94.0 (53.9-182.2)	-3.3	**0.0005**	96.8 (55.0-154.6)	94.6 (53.9-180.5)	-2.2	**0.003**	98.1 (62.5-206)	93.5 (58.8-182.2)	-4.6	0.059
Metabolic												
HbA_{1c}, mmol/mol	49 (25-124)	47 (22-110)	-2	**0.0045**	59 (35-124)	55 (33-110)	-4	**0.011**	38 (25-46)	36 (22-48)	-2	**0.012**
Total cholesterol, mmol/L	4.7 (2.4-9.0)	4.0 (1.9-8.1)	-0.7	**0.0023**	4.1 (2.4-7.7)	3.9 (1.9-8.1)	-0.2	**0.0071**	5.1 (2.7-9.0)	4.8 (2.7-7.1)	-0.3	0.13
HDL, mmol/L	1.0 (0.5-2.4)	1.0 (0.6-1.9)	0	0.75	1.0 (0.5-1.7)	1.0 (0.6-1.9)	0	0.85	1.0 (0.8-2.4)	1.1 (0.7-1.9)	0.1	0.57
Triglyceride, mmol/L	2.1 (0.67-17.0)	1.9 (0.6-9.0)	-0.2	0.28	2.1 (0.7-7.8)	2.0 (0.6-9.0)	-0.1	0.36	2.0 (0.7-17.0)	1.7 (0.7-7.4)	-0.3	0.6
Systolic blood pressure, mm Hg	140 (105-189)	135 (102-193)	-5	0.24	141 (105-189)	136 (102-193)	-5	0.76	138 (108-186)	134 (103-190)	-4	0.13
Liver stiffness												
Fib-4 score	1.1 (0.2-7.3)	1.2 (0.2-9.3)	0.05	0.71	1.5 (0.4-7.3)	1.2 (0.4-9.3)	-0.3	0.94	1.06 (0.23-4.88)	1.09 (0.21-3.99)	0.02	0.58
NFS	-1.0 (-6.5-+5.8)	-0.7 (-6.0-+3.9)	0.3	**0.0012**	0.02 (-3.9--3.0)	-0.3 (-4.7--3.1)	-0.3	0.089	-2.1 (-6.5-5.78)	-1.7 (-6.0-3.9)	0.4	**0.003**
Transient elastography, kPa	9.1 (3.5-75)	7.8 (3.2-57)	-1.3	**0.0097**	9.7 (4.3-75)	8.4 (4.4-57)	-1.3	0.067	8.8 (3.5-34)	6.8 (3.2-45.7)	-2.0	0.07
***CVD** (QRISK3)*												
Absolute risk, %	12.5 (0.1-60.9)	12.7 (0.1-52.6)	0.2	0.17	18.3 (1.7-61.0)	19.3 (1.9-53.0)	1.0	0.13	3.0 (0.1-21.6)	3.0 (0.1-18.1)	0.0	0.84
Relative risk	2.1 (0.7-11.5)	2.0 (0.8-12.9)	-0.1	**0.0001**	2.7 (1.1-11.5)	2.6 (1.1-12.9)	-0.2	**0.0006**	1.4 (0.7-5.3)	1.2 (0.8-6.2)	-0.2	0.066

Δ, difference between median at baseline (base.) and latest visit. Bold *P* value represents statistical significance. Wilcoxon signed rank test used between baseline and latest.

Figure 2.4: Change in weight and change in cardio-metabolic and liver health stratified by weight loss: baseline to latest follow-up

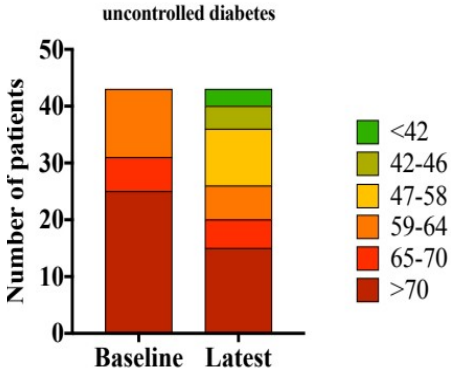


Data shown: Mean + Standard Error of Mean

Figure 2.5: Change in HbA1c in patients with Type 2 Diabetes Mellitus: baseline to latest follow-up

(a). Patients with poorly controlled T2DM (HbA1c > 58 mmol/mol) at baseline

(b). All patients with T2DM at baseline



Additional analyses of subjects by fibrosis stage (early fibrosis and advanced fibrosis) and by weight response (weight gain or weight loss) were also performed to gain an insight into improvements in liver and metabolic health. These results are summarised in Appendix 7.1.

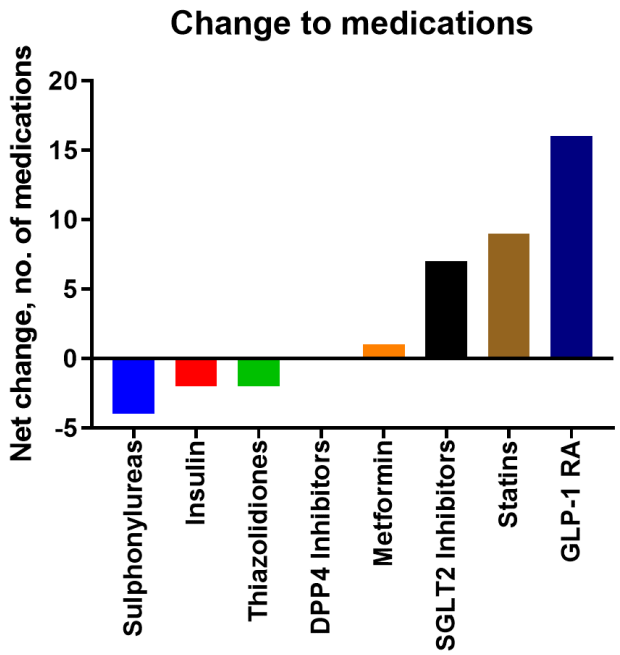
2.3.3 Changes in liver and cardio-metabolic health in patients with T2DM

As T2DM is strongly associated with NAFLD progression (29), a subgroup analysis of all patients with T2DM was performed. At baseline, patients with T2DM were older than patients without T2DM ($p<0.0001$) and, as expected, had higher baseline HbA1c ($p<0.0001$) (Table 2.1). Liver stiffness was also significantly higher at baseline in those with T2DM ($p=0.011$). There was a significant relationship between the presence of T2DM and total number of metabolic syndrome components ($p<0.0001$). Those with T2DM had lower total cholesterol at baseline than those without ($p=0.0011$) (Table 2.1) though statin use at baseline was higher in those with T2DM (65%) compared to those without T2DM (42%).

Median HbA1c reduced significantly by 4 mmol/mol between baseline to last follow-up in those patients with T2DM (Table 2.2). Improvement was most marked in patients who had poorly controlled T2DM at baseline (HbA1c >58 mmol/mol, n=43), reducing by 14 mmol/mol (Appendix 7.1). There were also significant improvements in categories of glycaemic control for those with poorly controlled T2DM subgroup (p = 0.0006) (Figure 2.4). Weight also decreased in patients with T2DM (Table 2.2).

At baseline, 85 (88%) of patients with T2DM were already prescribed oral glucose lowering therapy, 26% were prescribed insulin therapy and 5% were prescribed GLP-1 therapy. 21% of patients with T2DM started GLP-1 therapy over the duration of the study and 20% of patients who were on insulin therapy at baseline had this discontinued. In those who had insulin therapy discontinued (n=5), median HbA1c reduced by 13 mmol/mol from this point to latest follow-up. 15% of patients were commenced on statin therapy. Net changes to medications in patients with T2DM are summarised in Figure 2.5

Figure 2.6: Net changes to medication prescriptions between baseline and follow-up in patients with T2DM at baseline



2.3.4 Economic analysis and cost effectiveness

The UKPDS-OM2 model was used to calculate changes in quality adjusted life expectancy (QALE) and cost effectiveness of the multidisciplinary approach for all patients with T2DM. This estimated no significant difference in mean life expectancy in the intervention group (clinic attendees) compared to the reference group (baseline measures carried forward) (Table 2.3). In those with poorly controlled T2DM, the intervention group had a significantly increased mean life expectancy of 24 days (95 % CI: +2 to +50 days) compared to the reference group. Similarly, mean QALE was significantly increased by 29 days (95 % CI: +6 to +55 days) suggesting that patients with poorly controlled T2DM managed through our multidisciplinary clinic approach had significant improvements in both overall and quality adjusted life expectancy.

The UKPDS-OM2 was also used to evaluate cost-effectiveness in patients with poorly controlled T2DM. Lifetime (total) costs were £30.0k in the clinic group and £29.5k in the reference group who did not attend the clinic, a mean difference of £0.5k. Lifetime quality-adjusted life years (QALYs) were 11 years in the clinic group and 10.9 years in the reference group, a difference of 0.1 years (29 days). The resulting incremental cost-effectiveness ratio (ICER, cost per QALY) was £6.1k (95% CI: £0.3k - £59.3k) with 91 % of model bootstrap runs falling below a cost per QALY threshold of £20,000.

Table 2.6: Economic analysis of patients with T2DM using the UKPDS Outcomes Model v0.2

	Clinic Group		Reference Group		Difference	
	Average	**95% CI**	**Average**	**95% CI**	**Average**	**95% CI**
Mean Life Expectancy	years		years		days	
All with T2DM	14.0	13.7-14.3	14.0	13.8-14.3	-9.8	-23.4-+1.4
Poorly controlled T2DM	14.0	13.8-14.3	13.9	13.7-14.2	23.8	1.9-50.3
Quality Adjusted Life Expectancy	years		years		Days	
All with T2DM	11.1	10.9-11.3	11.1	10.9-11.3	-6.1	-18.2-+4.4
Poorly controlled T2DM	11.0	10.9-11.3	10.9	10.8-11.2	28.9	6.4-55.1
Therapy Costs, £						
All with T2DM	570	569-570	-	-	-	-
Poorly controlled T2DM	570	569-570	-	-	-	-
Complication costs, £k						
All with T2DM	30.3	29.4-31.5	30.2	29.5-31.3	0.03	-0.22-+0.38
Poorly controlled T2DM	29.4	28.4-30.8	29.5	28.7-30.5	-0.09	-0.53-+0.67
Total cost, £k						
All with T2DM	30.8	30.0-32.0	30.2	29.5-31.3	0.60	0.35-0.95
Poorly controlled T2DM	30.0	29.0-31.4	29.5	28.7-30.5	0.48	0.046-1.2
ICER (Cost per QALY), £k						
All with T2DM	-	-	-	-	-	-
Poorly controlled T2DM	6.1	0.3-59.3*	-	-	-	-
T2DM Cohort: n=97; Poorly Controlled T2DM Cohort: n=43 Clinic values held constant from year 3 onwards (group 1) vs. baseline values held constant from year 0 (group 2). Model parameters: 1 year of therapy at £591. 20,000 loops with 250 bootstraps run 5 times. Discount rate 3.5. *Percentage of bootstraps below Cost per QALY value of £20,000 = 91.2%.						

2.4 Discussion

Current UK and European guidelines (104,149), advise that management of NAFLD should be multifaceted and patients should be managed using a multidisciplinary approach (147,264) which is particularly important given that there are no directly licenced therapies as yet. Nevertheless, there is still a paucity of best practice data describing how such

services should be shaped and delivered as well as objective evaluations of the impacts of a multidisciplinary approach on patient outcomes.

In this chapter, I have described and evaluated the management of a cohort of patients with NAFLD via a dedicated multidisciplinary Metabolic Hepatology clinic. This demonstrated significant improvements in both clinical and surrogate markers of liver and cardio-metabolic health over a 13-month median follow-up period. This was an observational study of real-life clinical data making it applicable to clinical practice. There was, however, no control arm and the intervention was not uniform for the cohort given the personalised nature of the clinical approach, so causality cannot necessarily be inferred. Moreover, such data may be confounded by selection bias and regression to the mean. Nevertheless, it forms a useful benchmark of current clinical practice to which other services or interventions may be compared.

2.4.1 Improvements in markers of liver health

There was a 14 % reduction in liver stiffness in patients attending the metabolic hepatology clinic, measured at point of care, and an associated 21 % improvement in serum ALT, an insensitive marker of NASH, though one which is still commonly used by healthcare professionals (105,106). This improvement in ALT is similar to that observed in patients with NAFLD managed through a similar multidisciplinary approach (264) and in lifestyle intervention trials (276). ALT has also been highlighted as a non-invasive surrogate marker of response in clinical trials in NAFLD/NASH (see chapter 4.4.1.2.1 for further details and discussion) (277,278). Further work is also need to assess other non-invasive biomarkers, serological or non-serological, for use in monitoring changes in liver disease severity or to predict liver-related health outcomes, mainly due to their poor sensitivity to detect small changes in liver fibrosis (29). In this cohort, there was no significant improvement in the Fib-4 score. There were also no significant changes in the numbers of patients predicted to have

advanced liver fibrosis between baseline and follow-up using non-invasive scores, though the relevance or utility of these findings is not known.

2.4.2 Improvement in glycaemic control

Both UK NICE (279) and American Diabetes Association (ADA) (280) guidance highlight the importance of 'personalised' or 'individualised' care when managing patients' diabetes taking into account comorbidities and include guidance on weight management. In particular, the more recent ADA guidance outlines that clinicians should consider the weight and cardiovascular risk effects of glucose lowering therapies when escalating diabetes therapy which is particularly important as some newer agents such as GLP-1 agonists and SGLT2 inhibitors have been shown to have beneficial effects on either or both of weight and cardiovascular risk (see Chapter 1). In the UK, GLP-1 therapy has a licence for use in patients with T2DM, and NICE guidance (279) outlines that this therapy is recommended in patients with poorly controlled T2DM who are obese, owing to improvements to glycaemic control and the promotion of weight loss.

It should also be noted that the drug pioglitazone licenced for the treatment of diabetes, has also been shown to be reduce hepatic steatosis and inflammation as well as having a possible improvement in fibrosis (see Table 1.3). The side effect profile of pioglitazone and other drugs of the same class has however generated considerable debate including concerns regarding weight gain, cardiovascular and bone fracture risk. Furthermore, during the dates on which this analysis is conducted, data which received high profile coverage was published suggesting a possible link between pioglitazone and bladder cancer which is another reason to account for why pioglitazone use was low in my analysis (281). Interestingly, more recent data has been reassuring regarding the bladder cancer association (282) and separate data suggests a possible beneficial cardiovascular effect of the drug (283).

My analysis indicates a reduction in prescriptions for diabetes medications, such as insulin, that are associated with weight gain and an increase in prescriptions for diabetes medications that are weight neutral or associated with weight loss and those which also potentially confer cardiovascular protection. In particular, initiation of GLP-1 therapy in patients with T2DM as per UK guidelines was the most common pharmacological intervention in the OUH clinic.

These finding were complemented by overall improvement in glycaemic control for all patients with T2DM, with improvement most marked in those with poorly controlled T2DM. These findings on improvement in glycaemic control in a real life clinic setting are consistent with previously published studies investigating the impact of diet and or exercise in patients with T2DM. For example, in the Early ACTID trial in patients with recently diagnosed T2DM who had good glycaemic control at baseline (mean baseline HbA1c 49-50 mmol/mol), HbA1c improved by around 3.3 mmol/mol at 6 and 12 months in those provided with 'dietary support' (dietary consultation every 3 months with monthly nurse support), compared with a control group provided with 'usual care' (initial dietary consultation and follow-up every 6 months – which is often the standard model and frequency of care for patients managed in primary care) (284). Additionally, in this study there was no difference in improvement in HbA1c between the 'dietary support' group and a 'diet plus activity' group (as per diet group, plus 30 min brisk walking five times per week) at either 6 or 12 months. Previous studies have however shown that a combination of diet and exercise is effective in improving glycaemic control by around 11 mmol/mol at 6 and 12 months (285). Similarly, in another study, patients with T2DM either provided with specifically tailored food for 1 year or with monthly individual dietary counselling advice saw an approximate 11 mmol/mol reduction in HbA1c over 12 months, whilst a control group receiving standard diabetes care monthly via appointment with a doctor or nurse saw no change in HbA1c over 12 months (286,287). Improvement in glycaemic control of the scale shown in the OUH clinic are accepted as likely to improve both diabetes-related and liver-related complications.

2.4.2 Improvement in weight

Overall, patients achieved a median 3.4 % weight loss, though there was considerable variation in response. 23 % of patients (37/159 with both baseline and latest weight measurements recorded) lost at least 5 % of their baseline weight by latest visit. Previous studies have demonstrated an association between weight loss and improvements in NAFLD, with a reduction in excess of 3-5 % thought to be required for improvement of hepatic steatosis and / or NASH (288,289).' Stratification of markers of liver and cardio-metabolic health by weight response demonstrated that improvements to these markers appear to correlate with degree of weight loss. My findings also suggest that even modest weight loss is associated with improvements in markers of liver and cardio-metabolic health. Given the relatively short duration of follow-up, the dynamics of weight loss and any subsequent weight gain were not analysed in this study but would comprise an important follow-up analysis.

Patients often struggle to lose and maintain weight loss without structured support. There is evidence that commercial weight loss programmes have significant benefit (290) with weight loss of >5 % reported, which is of a magnitude that will confer benefit to patients with NAFLD. The GLP-1 agonist Liraglutide at a higher dose of 3mg daily is also licenced for use in patients with obesity without diabetes, but its use in this context is not yet supported through NICE guidance within the UK NHS.

2.4.2 Economic analysis

The simulation of the lifetime costs and outcomes of the clinic intervention in patients with poorly controlled T2DM indicated that it had a high probability of being clinically effective with

increased quality adjusted life expectancy and cost-effective at the thresholds used by NICE, with an incremental cost per QALY of £6.1k. Whilst there was no dedicated control group available, a standardised modelling approach was nevertheless adopted.

These findings provide useful additional information and have significance due to the high and growing burden of NAFLD and the need for health authorities to have both clinically effective and cost-effective strategies at their disposal when working within confined financial envelopes. Given that GLP-1 therapy is relatively safe and well tolerated by patients and was found to be an important feature of practice within the OUH service, these findings may also help support any future economic justification and pave the way for GLP-1 therapy being made available to patients with NAFLD without T2DM within the NHS. particularly as this class of agent already has a licence for weight loss in patients without T2DM.

2.5 Conclusion

While considerable effort is going into the development of novel therapies that target the hepatic manifestations of NAFLD/NASH, data from this study indicate that significant improvements in surrogate markers of liver and cardio-metabolic disease can nevertheless be achieved through effective implementation of existing risk-stratification and treatment strategies. Given that NAFLD is both a liver and a cardio-metabolic disease, this analysis has shown that adopting a multidisciplinary approach to its management - involving not only hepatologists, diabetologists and metabolic physicians, but also allied health professionals including diet and lifestyle experts - is a clinically effective and potentially cost-effective way to manage these complex patients. Furthermore, the findings have shown that GLP-1 therapy has significant appeal and benefit in its use in patients with NAFLD and T2DM due to favourable impact on weight and glycaemic control and in their proven cardiovascular benefits. Due to these benefits as well as its favourable side effect profile, arguments for its use to be widened to patients with NAFLD without T2DM appear to be strong.

The following 2 research chapters will explore the 2 highlighted themes in this chapter further. Firstly, they will explore novel risk stratification tools for patients with NAFLD and secondly investigate the use of GLP-1 agonists on NAFLD as a potential new licenced therapy in separate clinical studies.

Chapter 3 The urinary steroid metabolome as a novel non-invasive assessment tool to stage and grade NAFLD severity

The material presented within this chapter was instigated and led by myself. The computational modelling data contained within are the result of an academic collaboration with the laboratory of Prof Michael Biehl and Mr Jasper De Boer at the University of Gronignen who have considerable expertise in machine learning. Mr Jasper De Boer and myself, with guidance from Prof Jeremy Tomlinson and Prof Michael Biehl, jointly devised the mathematical experiments which were subsequently analysed using the machine learning algorithms outlined in the chapter. The final GMLVQ analyses presented in this chapter were run by Mr Jasper De Boer on steroid data processed and prepared for analysis by myself and after agreement on the final design from the four persons outlined.

3.1 Introduction

3.1.1 Risk stratification in NAFLD

As outlined in Chapter 1, despite the adverse clinical outcomes associated with NAFLD, it is often asymptomatic until its late stages when either liver failure or cardiovascular complications become apparent. Accurate and early staging is therefore crucial to determine patient risk of complications and to guide the most appropriate management strategy and whether this occurs in primary or secondary care.

The current gold standard for staging liver fibrosis in patients with NAFLD remains a liver biopsy, which is invasive, associated with morbidity, resource intensive and samples only a very small fraction of the liver and therefore may be prone to error (110). Faced with this challenge, several non-invasive tools, including serological, clinical and imaging based markers and algorithms have been developed in order to try and reduce the need for liver biopsy to stage fibrosis in NAFLD (1). Imaging modalities include magnetic resonance elastography and multi-parametric magnetic resonance imaging (MRI) as well as transient hepatic elastography (142,291). Similarly, algorithms of varying complexity, which

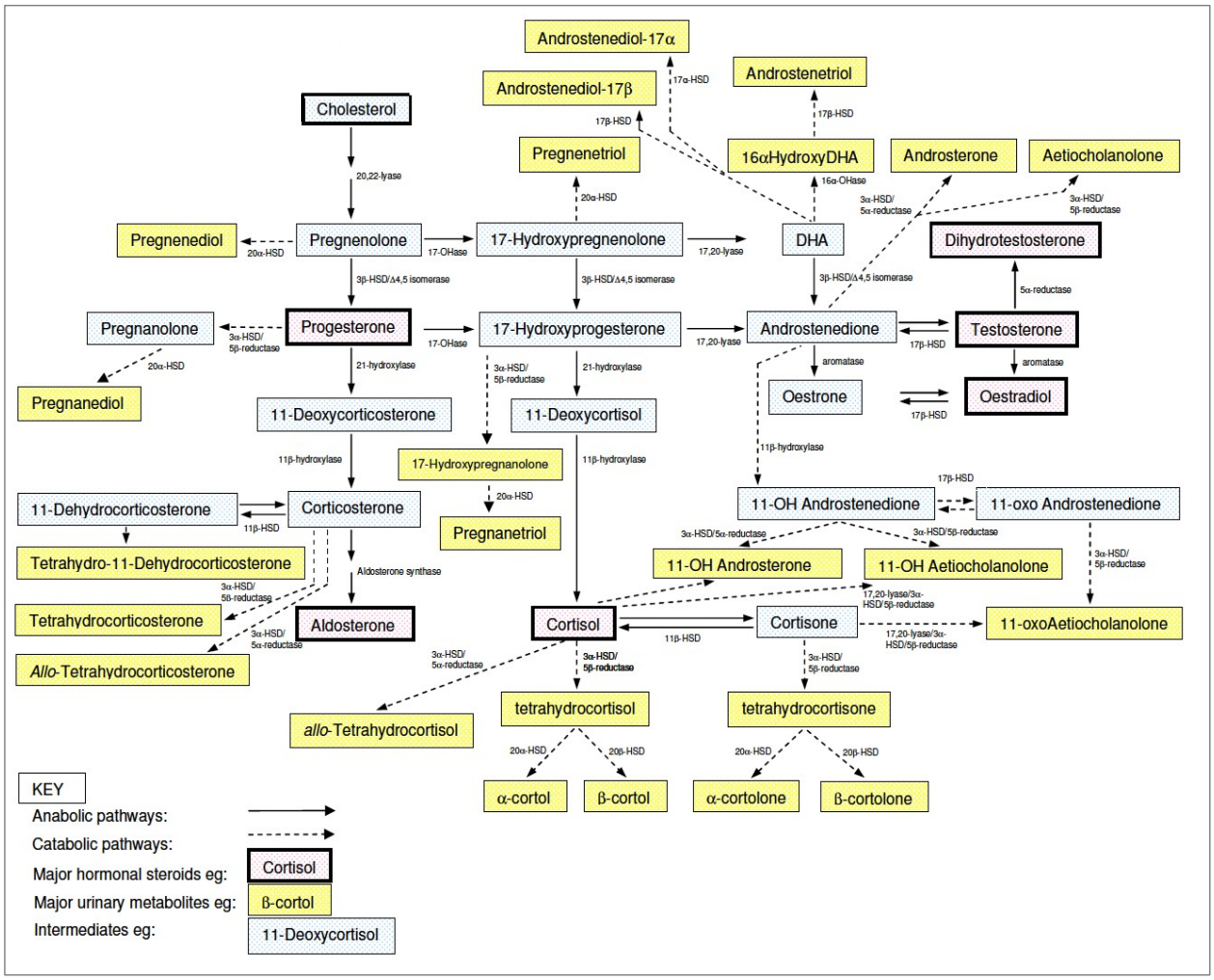
incorporate both serological and clinical markers, for example the FIB-4 score, NAFLD Fibrosis Score and Enhanced Liver Fibrosis score (ELF) are often used to help stratify patients as being at high risk of advanced liver fibrosis (118,120,124) above and beyond use of routine liver biochemistry which has limited utility in helping to stage NAFLD (292). Nevertheless, to date, no existing non-invasive techniques have been shown to be sufficiently robust to replace liver biopsy in clinical practice. In general, most of these approaches have good negative predictive value, however, sensitivity and positive predictive value are relatively poor. Please see Chapter 1 for a more detailed review of current non-invasive risk stratification modalities used in clinical practice.

3.1.2 Steroid hormone metabolism and NAFLD

Steroid hormones – including glucocorticoids, mineralocorticoids and androgens, are primarily synthesised in the adrenal glands and gonads. However, the majority of their metabolism (often to inactive metabolites) occurs within the liver with subsequent excretion in the urine. Based upon the paradigm of glucocorticoid excess (Cushing's syndrome), in which patients develop a florid metabolic phenotype characterized by obesity, insulin resistance, T2D and NAFLD (98), previously published work has investigated specific pathways of glucocorticoid metabolism to determine if these are dysregulated in patients with NAFLD. As examples, the activities of the enzymes 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which regenerates cortisol (F) from inactive cortisone (E), and the A-ring reductases (5α- and 5β-reductase, 5αR/5βR), which inactivate cortisol to tetrahydrocortisol metabolites (5αTHF and THF), have been shown to be dysregulated in patients with NAFLD (as determined by specific steroid hormone metabolite ratios measured in urine) (100,293). However, these studies have been small and very few have tried to examine the impact of histological stage and disease progression. In addition, the complexities of steroid hormone metabolism within the liver mean that specific ratios are influenced by the activities of multiple enzymes and therefore their interpretation is challenging. Figure 3.1 provides a

summary outline of steroid hormone biology and metabolism showing the major steroid hormones and associated metabolites as well as the steroid metabolising enzymes governing steroid hormone metabolism.

Figure 3.7: A schematic summary of steroid hormone biology and metabolism



3.1.3 Hypothesis

The research in this chapter therefore tests the hypothesis that the urinary steroid metabolome provides an accurate and dynamic reflection of steroid hormone metabolism within the liver, and that this will be influenced by NAFLD disease stage in a sensitive and specific manner. Through the adoption of interpretable machine learning algorithms, which

simultaneously permits specific pathway interrogation as well as a global analysis, this work investigates whether the urinary steroid metabolome may offer potential to accurately and non-invasively diagnose and stage NALFD.

3.2 Methods

3.2.1 Subject selection

Clinical data and urine samples (spot or 24 hour collections) were collected from 227 subjects including from 121 patients with NAFLD and 106 from controls without known liver disease. All patients with NAFLD had liver biopsy staging performed, except in 6 patients where a diagnosis of cirrhosis was made using established clinical criteria (clinical examination, platelets and liver function blood tests, imaging, elastography where appropriate). Determination of control status was established by review of medical history and the absence of any known liver disease. Control subjects with abnormal liver chemistry or with elevated non-invasive serum fibrosis assessments (see below) were excluded from the analysis. Where data in individual subjects was available, scores for non-invasive markers of liver fibrosis were calculated (120). These were defined as follows:

- APRI (AST to Platelet Ratio Index) = AST (IU/L) / (upper limit of normal) / platelet count ($\times 10^9$/L) × 100
- FIB-4 (Fibrosis-4 score) = age × AST (IU/L) / platelet count ($\times 10^9$/L) × √ALT (IU/L)
- AST/ALT ratio = AST (IU/L) / ALT (IU/L)
- NAFLD fibrosis score = −1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m^2) + 1.13 × Impaired fasting glucose or T2D (yes = 1, no = 0) + 0.99 × AST/ALT ratio −0.013 × platelet count ($\times 10^9$/L) −0.66×albumin (g/dL)
- BARD score = sum (BMI>28kg/m^2 = 1, AST/ALT ratio>0.8 = 2, T2D = 1)

3.2.2 Histological liver staging of NAFLD

Liver biopsies were performed as part of routine clinical care in patients with NAFLD. NAFLD Activity Score (NAS) (including the individual components of lobular inflammation, steatosis, hepatocyte ballooning and fibrosis) as well as NAFLD fibrosis stage (F0-F4) was assessed by the Kleiner scoring system (108). F0 represents the absence of fibrosis, F1 portal or perisinusoidal fibrosis, F2 portal/periportal and perisinusioidal fibrosis, F3 septal or bridging fibrosis and F4 cirrhosis.

3.2.3 Urinary steroid metabolome analysis

3.2.3.1 Steroid extraction and GC-MS analysis

Urine samples were collected and stored at -80^{0}C. Measurement of urinary steroid metabolites was undertaken using gas chromatography / mass spectrometry (GC-MS) at the University of Birmingham by research scientists using a standardised method as previously reported (294). In brief, free and conjugated steroids were extracted from 1 mL of urine via a 5-step extraction method. Solid-phase extraction of free and conjugated steroids was performed. Steroid conjugates underwent enzymatic hydrolysis followed by solid-phase re-extraction of steroids, chemical derivatization to form ethers, and finally liquid-liquid extraction.

GC-MS was undertaken on an Agilent 5973 MSD single quadrupole gas chromatography mass spectrometer (Agilent, Santa Clara, USA) instrument allowing quantification of up to 32 steroid metabolites, with representation of major steroids and their metabolites from all the adrenally derived steroid hormone classes (androgens, glucocorticoids and mineralocorticoids (Table 3.1). Steroids were identified in SIM (single ion monitoring) mode and quantified relative to authentic reference standards.

3.2.3.2 Urinary creatinine assay

For the GC-MS analysis, for each urine sample, a urinary creatinine correction was made in an attempt to adjust for differing times and durations of collection as urinary creatinine is excreted at a relatively constant rate and is widely used as a corrective factor in the analysis of urine metabolites (295). These data were expressed as µg steroid/g urinary creatinine.

Urinary creatinine measurement was performed by myself using the QuantiChromTM Creatinine Assay Kit (DICT-500, Universal Biologicals, UK). 5 µL of either standard (50 mg/dL) or urine were mixed with 200 µL of working reagent in a 96-well plate. Optical density (OD) was read at 0 min and 5 min at an absorbance of 490 nm on a VersaMax Plate Reader (Molecular Devices, UK) and the creatinine concentration (mg/dL) was calculated for each urine sample in duplicate as per manufacturer guidance. A mean creatinine value (mg/dL) was calculated from a minimum of 2 independent assays.

A separate analysis of urinary steroid data that was not corrected for urinary creatinine is also presented and is expressed as µg steroid /1000 mL urine.

3.2.3.3 Steroid extraction and LC-MS/MS analysis

Measurement of 26 of the 32 GC-MS urinary steroid metabolite panel (see Table 3.1) was repeated in a subset of 77 patients with established NAFLD using a high throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) platform at the University of Birmingham. Steroids in urine were deconjugated from their sulphate and glucuronide esters prior to analysis. Deconjugation was conducted by heating the urine at 60°C for 3 hours in a preparation of helix pomatia (0.2 M acetate buffer with 10 mg sulphate and 10 mg ascorbate/3 mL, Sigma Aldrich, UK). The hydrolysed mixture was subsequently extracted by solid phase extraction using a 96 well plate of 100 mg C18 columns (Biotage, UK). Columns were activated with methanol, then washed with water, sample was applied to the cartridge

and the sample was washed again with water. Following this, steroids were eluted from the column with 1 mL of methanol and evaporated to dryness under nitrogen at 60°C. This extraction method has previously been described and published (296).

For LC-MS/MS analysis, a water/methanol gradient system with 0.1% formic acid was used to separate the steroids on a HSS T3, 1.8µm, 1.2x50mm column (Waters, UK). A Waters Xevo-XS triple quadrupole mass spectrometer with electrospray ionisation mode was utilised in positive ionisation mode attached to an Acquity ultra-high pressure liquid chromatography system (Waters, UK). For positive identification and quantitation steroids were compared to authentic reference standards prepared in synthetic urine. A matching retention time and two matching mass transitions (MRMs) were required for positive identification. A calibration series for each steroid was prepared ranging from 0.5 to 3000 ng/mL with inclusion of a blank. Each steroid was quantified relative to a specific internal standard.

Table 3.7: Numerical values assigned to individual steroids and metabolites measured using GC-MS

[#]Urinary steroid metabolites also analysed using LC-MS/MS in addition to GC-MS. In addition, the sum of 'THA +5aTHA' was used as 1 metabolite in the LC-MS analysis.

No.	Common name	Chemical name
1	Androstendione[#]	5α-androstan-3α-ol-17-one
2	Etiocholanolone[#]	5β-androstan-3α-ol-17-one
3	11β-hydroxyandrosterone[#]	5α-androstane-3α, 11β-diol-17-one
4	Dehydroepiandrosterone[#]	5-androsten-3β-ol-17-one
5	16α-hydroxy-dehydroepiandrosterone	5-androstene-3β,16α-diol-17-one
6	Pregnenetriol[#]	5-pregnene-3β,17, 20α-triol
7	Pregnenediol[#]	5-pregnene-3β, 20α-diol *and* 5, 17, (20)-pregnadien-3β-ol
8	Tetrahydro-11-	5β-pregnane-3α, 21-diol, 11, 20-dione

	dehydrocorticosterone	
9	5α-tetrahydro-11-dehydrocorticosterone	5α-pregnane-3α, 21-diol-11, 20-dione
10	Tetrahydrocorticosterone[#]	5β-pregnane-3α,11β,21-triol-20-one
11	5α-tetrahydrocorticosterone[#]	5α-pregnane-3α, 11β,21-triol-20-one
12	18-hydroxytetrahydro-11-dehydrocorticosterone	5β-pregnane-3α, 3,18,21-trihydroxy-11,20-dione
13	Tetrahydro-11 deoxycorticosterone[#]	5β-pregnane-3α, 21-diol-20-one
14	Tetrahydroaldosterone[#]	5β-pregnane-3α, 11β,21-triol-20-one-18-al
15	Pregnanediol[#]	5β-pregnane-3α, 20α-diol
16	3α,5α-17-hydroxypregnanolone	5α-pregnane-3α, 17α-diol-20-one
17	17-hydroxypregnanolone[#]	5β-pregnane-3α, 17α-diol-20-one
18	Pregnanetriol[#]	5β-pregnane-3α, 17α,20α-triol
19	Pregnanetriolone[#]	5β-pregnane-3α, 17,20α-triol-11-one
20	Tetrahydro-11-deoxycortisol[#]	5β-pregnane-3α, 17, 21-triol-20-one
21	Cortisol[#]	4-pregnene-11β, 17, 21-triol-3, 20-dione
22	6β-hydroxy-cortisol	4-pregnene-6β, 11β, 17, 21-tetrol-3, 20-dione
23	Tetrahydrocortisol[#]	5β-pregnane-3α, 11β,17, 21-tetrol-20 one
24	5α-tetrahydrocortisol[#]	5α-pregnane-3α, 11β,17, 21-tetrol-20-one
25	α-cortol	5β-pregnan-3α, 11β, 17,20α, 21-pentol
26	β-cortol[#]	5β-pregnan-3α, 11β, 17, 20β, 21-pentol
27	11β-hydroxyetiocholanolone[#]	5β-androstane-3α, 11β-diol-17-one
28	Cortisone[#]	4-pregnene-17α, 21-diol-3, 11, 20-trione
29	Tetrahydrocortisone[#]	5β-pregnene-3α, 17, 21-triol-11, 20-dione
30	α-cortolone[#]	5β-pregnane-3α, 17,20α, 21-tetrol-11 one
31	β-cortolone[#]	5β-pregnane-3α, 17,20β, 21-tetrol-11-one
32	11-oxoetiocholanolone[#]	5β-androstan-3α-ol-11,17-dione

3.2.3 Steroid Hormones and ratios

Measurement of individual steroid hormone concentrations and their metabolites permitted assessment of individual steroid metabolic pathways based upon the analysis of 'precursor metabolite to product metabolite' ratios. This approach is well established and allows the assessment of specific enzymatic activities (294). All individual steroid data was log transformed (Log10) prior to analysis. Product to pre-cursor metabolite ratios investigating specific pathways of glucocorticoid metabolism were calculated as follows:

- ***Total Cortisol (F) Metabolites*** = *6β-hydroxy-cortisol + tetrahydrocortisol (THF) + 5α-tetrahydrocortisol (5αTHF) + α-cortol + β-cortol + 11β-hydroxyetiocholanolone + cortisone (E) + tetrahydrocortisone (THE) + α-cortolone + β-cortolone + 11-oxoetiocholanolone*
- ***11β-HSD1 activity*** = *(THF+5αTHF) / THE*
- ***A-ring reductase activity*** = *5αTHF / THF*

3.2.4 Generalized matrix learning vector quantization (GMLVQ) computational analysis

Learning Vector Quantization (LVQ) is a machine learning technique that extracts typical class representatives or prototypes from training data (297). For this analysis, this translated to one typical steroid profile per disease stage. These prototypes can be used to classify a steroid profile with unknown disease stage: the most probable disease stage is determined by selecting the class of the prototype that is most similar to the new profile. The dis-similarity of a given steroid profile and a prototype is defined by a distance measure, for example the conventional Euclidean distance. In Generalized Matrix Learning Vector Quantization (GMLVQ) (298), however, the distance metric itself is adaptive and optimized together with the prototypes in the same data driven training process. This metric is defined

through a matrix of adaptive parameters, termed the relevance matrix. Its diagonal elements quantify the importance of individual steroids in the classification scheme.

GMLVQ analysis of GC-MS data was designed by Dr Ahmad Moolla, Prof Michael Biehl and Mr Jasper De Boer and performed by Mr Jasper De Boer in subjects who provided a spot urine sample using a panel of 32 steroids. Subject records with missing steroid measurements were removed prior to analysis. A further subselection was made to compare GMLVQ analysis and the non-invasive scores on a unified set: records with missing BMI, diabetes, ALT, AST, albumin or platelets in the F0-F4 groups were removed. Records of 128 patients remained in the set (control=56, F0-F2=19, F3-F4=53).

Steroid data was log transformed (Log10) before undergoing standardisation by z-score transform during the GMLVQ analysis. Remaining missing values for age and BMI in the control group were treated along the lines of the NaN-LVQ prescription, ignoring them in the computation of the corresponding distances (299).

Feature selection was used to refine the model to investigate the performance of a reduced number of steroids. The top 10 most relevant steroids were identified from the relevance matrix to reduce the steroid number from 32 to 10. Following this, a backwards elimination ‘greedy search’ strategy was employed to reduce the number of steroids from 10 to 2 sequentially which involved re-training the GMLVQ system each time the least relevant steroid was removed.

Due to the sample size of the cohort, repeated random sub-sampling validation (300) was applied to divide the dataset into training and validation sets in order to evaluate GMLVQ performance. The process was repeated to produce 200 results, each one corresponding to a division of 90 % for training and 10 % for validation. The randomized sets were stratified in that both training and validation sets contained at least one example from each class.

Receiver operating characteristics (ROC) (300,301) and area under curve (AUC) of the ROC curve was used as the primary performance metric to compare newly generated models and

various alternative established non-invasive scores for liver fibrosis. Bootstrapping (300) was used to calculate 95 % confidence intervals for the mean ROC values and mean feature relevances. 10,000 bootstrap samples were taken from the 200 validation results. Mean values per sample were calculated and the borders of the centre 95 % values were used to provide the confidence interval.

3.2.5 Statistical analysis

Steroid metabolite ratio data is graphically represented as mean and standard error of the mean using GraphPad Prism version 7.02 (GraphPad Software, California). Individual steroid data and steroid ratios were compared between control, early fibrosis and advanced fibrosis groups using the Kruskal-Wallis non-parametric test and pair-wise multiple comparisons between groups were undertaken using Dunn's post hoc test. Statistical analysis performed on log transformed steroid values or ratios. Significance was determined as $p<0.05$.

3.3 Results

3.3.1 Subject demographics

227 individuals were recruited into the study. Demographic details as well as biochemical and histological assessment are presented in Table 3.2. There was no significant difference in gender ratios between groups. Age was significantly different between all 3 groups. Those with advanced fibrosis were older than the other two groups though controls were older than those with early fibrosis (F0-F2). BMI was significantly different across all groups; all groups had a mean BMI in the obese range (BMI >30 kg/m^2) with those with early fibrosis being the most obese. The prevalence of T2DM significantly increased with increasing fibrosis stage with 63% of subjects with advanced fibrosis having a diagnosis of T2DM. ALT was significantly higher in both early and advanced fibrosis groups compared with control group though there was no significant difference between early and advanced fibrosis groups. There was a trend to a higher proportion of those with NASH in the advanced fibrosis group, though this did not reach significance (p=0.07).

*Table 3.8: Demographic details of study participants***

	Control	F0-2	F3-4	p-value
N (m/f) (males, %)	106 (41/65) (38.7)	39 (20/19) (51.3)	82 (39/43) (47.6)	0.29
Age, years	55.5 ± 11.1	45.6 ± 12.0*	61.8 ± 10.8*§	<0.0001
BMI, kg/m^2	30.7 ± 5.8	38.5 ± 7.0*	33.7 ± 5.8*§	<0.0001
Proportion with Type 2 Diabetes, %	3.8	30.8*	63.4*§	<0.0001
HbA1c, mmol/mol	38.6 ±10.4	40.8 ± 8.2	50.0 ± 13.5*§	<0.0001
Platelets, 10^9/L	n/a	242.5 ± 64.2	183.9 ± 67.0§	<0.0001
ALT, IU/L	13.2 ± 8.7	63.4 ± 51.4*	49.9 ± 36.9*	<0.0001
AST, IU/L	n/a	34.4 ± 22.0	49.1 ± 31.8§	0.0006
Fib-4 Score	n/a	0.93 ± 0.7	2.61 ± 1.7§	<0.0001
NAFLD Fibrosis Score	n/a	1.9 ± 1.2	3.8 ± 1.6§	<0.0001
NAS Score (0-8)	n/a	4.0 ± 1.7	4.7 ± 1.3§	0.029
Proportion with NASH (NAS Score>4, %)	n/a	42.1	63.1	0.07

***227 subjects: 106 controls and 121 individuals with biopsy-proven NAFLD stratified by fibrosis stage (F0-2 vs. F3-4). Data expressed are mean ± standard deviation (unless otherwise stated). Where applicable, p-value stated in the final column is the summary ANOVA value when all 3 groups are compared. (* p<0.05 vs. control; § p<0.05 vs. F0-2)*

3.3.2 Increased 11β-hydroxysteroid dehydrogenase type 1 and 5α-reductase activity in patients with advanced NAFLD

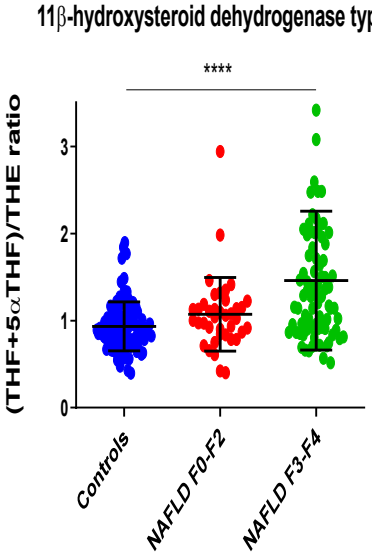
Previous studies in small numbers of patients (often without liver biopsy) have identified specific changes in urinary steroid metabolites ratio (100,293). In this cohort, the (THF+5αTHF)/THE ratio reflecting 11β-HSD1 activity was increased, consistent with enhanced cortisol regeneration in patients with advanced NAFLD (Figure 3.2a), though not

in those with mild disease (F0-2). In parallel, there was an increase in 5α-reductase activity, which would enhance cortisol clearance (Figure 3.2b). There was no change in total glucocorticoid metabolite production (Figure 3.2c). Data for specific steroid metabolites and ratios indicative of specific enzyme activity are presented in Table 3.3.

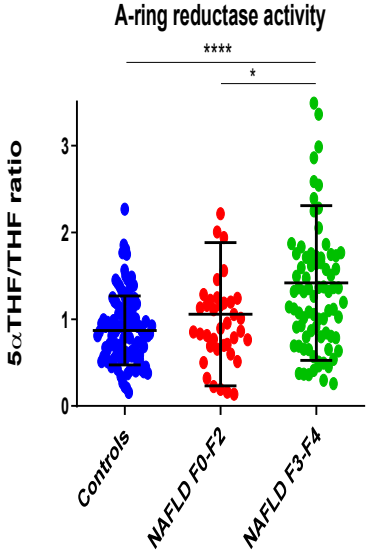
Figure 3.8: Total glucocorticoid metabolites, 11β-hydroxysteroid dehydrogenase type 1 and 5α-reductase activity

*Data shown: mean ± SD. 2 and 4 data points not shown in (a) and (b) respectively for graphical purposes. Both 11β-hydroxysteroid dehydrogenase type 1 (a) and 5α-reductase (b) activity are increased in patients with NAFLD with advanced fibrosis, although not in those with mild disease when compared to controls. Total glucocorticoid metabolite production was not different across the spectrum of NAFLD or in comparison with controls (**** p<0.0001, * p<0.05).*

a


11β-hydroxysteroid dehydrogenase type 1
****
(THF+5αTHF)/THE ratio
0
1
2
3
Controls
NAFLD F0-F2
NAFLD F3-F4

b


A-ring reductase activity
****
*
5αTHF/THF ratio
0
1
2
3
Controls
NAFLD F0-F2
NAFLD F3-F4

c

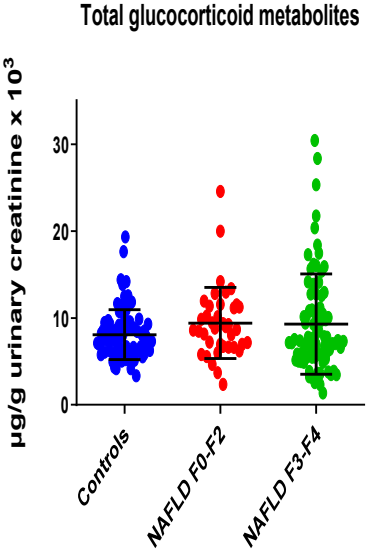

Total glucocorticoid metabolites
µg/g urinary creatinine x 10³
0
10
20
30
Controls
NAFLD F0-F2
NAFLD F3-F4

Table 3.9: Urinary steroid metabolite analysis

*Analysis of GC-MS data on spot urine samples from 106 control subjects and 121 with NAFLD stratified by fibrosis stage. (THE = tetrahydrocortisone, THF = tetrahydrocortisol, UFF = urinary free cortisol, UFE = urinary free cortisone, 11OH-androst = 11hydroxyandrosterone, 11OH-etio = 11hydroxyetiocholanolone, 11oxo-etio = 11oxo-etiocholanolone, Total glucocorticoid metabolites = cortisol+6β-OH-Cortisol+THF+5αTHF+α-cortol+β-cortol+11b-OH-ETIO+ cortisone+THE+α-cortolone+β-cortolone+11-oxo-etio, Fm = cortisol+THF+5αTHF+α-cortol+β-cortol, Em = cortisone+THE+α-cortolone+β-cortolone). Statistical analysis was performed on log transformed steroid values or ratios, * p<0.05 vs. control; § p<0.05 vs. F0-2.*

	Control	NAFLD F0-2	NAFLD F3-4
Corticosteroid metabolites, μg/g urinary creatinine (mean ± SEM)			
Androstendione	959 ± 53	1787 ± 328	835 ± 81*§
Etiocholanolone	918 ± 56	1029 ± 121	466 ± 55*§
11β-hydroxyandrosterone (11OH-androst)	442 ± 21	630 ± 87	642 ± 46*
Dehydroepiandrosterone (DHEA)	249 ± 44	428 ± 103	139 ± 39*§
16α-hydroxy-dehydroepiandrosterone	287 ± 31	347 ± 54	387 ± 49
Pregnenetriol (5-PT)	156 ± 13	284 ± 37*	165 ± 20§
Pregnenediol (5-PD)			
Tetrahydro-11-Dehydrocorticosterone (THA)	97 ± 7	101 ± 9	83 ± 9*§
5α-tetrahydro-11-Dehydrocorticosterone (5αTHA)	87 ± 3	80 ± 7	60 ± 7*§
Tetrahydrocorticosterone (THB)	101 ± 8	107 ± 11	98 ± 14§

5α-tetrahydrocorticosterone (5αTHB)	219 ± 14	296 ± 31	217 ± 24§
18-hydroxytetrahydro-11-Dehydrocorticosterone	44 ± 3.	46 ± 4	57 ± 5
Tetrahydro-11 deoxycorticosterone (TH-DOC)	14 ± 1	12 ± 1	9 ±1*§
Tetrahydroaldosterone (3a5bThaldo)	30 ± 2	26 ± 2	43.7 ± 4*§
Pregnanediol (PD)	161 ± 16	132 ±17	128 ± 29*
3α,5α-17-hydroxypregnanolone	9 ± 1	15 ± 2	12 ± 1
17-hydroxypregnanolone	85 ± 7	89 ± 9	79 ± 11§
Pregnanetriol (PT)	323 ± 16	316 ± 24	234 ± 19*§
Pregnanetriolone	25 ± 6	16 ± 2	15 ± 2§
Tetrahydro-11-deoxycortisol (THS)	68 ± 5	65 ± 7	80 ±10
Cortisol	56 ± 6	87 ± 14*	139 ± 16*§
6β-hydroxy-cortisol	94 ± 5	122 ± 19	161 ± 16*
Tetrahydrocortisol (THF)	1389 ± 69	1583 ±130	1512 ± 145
5α-tetrahydrocortisol (5αTHF)	1114 ± 54	1669 ± 211	1682 ±126*
α-cortol	269 ±14	334 ± 23*	406 ± 35*
β-cortol	380 ± 20	381 ± 25	434 ± 28
11β-hydroxyetiocholanolone (11OH-etio)	228 ± 16	122±16*	123 ± 13*
Cortisone	78 ± 4	104 ± 14	191 ± 17*§
Tetrahydrocortisone	2835 ± 130	3021 ± 223	2607 ± 225§
α-cortolone	1130 ± 52	1297 ± 79	1282 ± 86
β-cortolone	551 ± 24	526 ± 30	620±44
11-oxoetiocholanolone (11oxo-etio)	296 ± 19	168 ± 16*	123 ± 12*
Total glucocorticoid metabolites	8072 ± 306	9415 ± 656	9282 ± 638
Total cortisol metabolites (Fm)	3208 ± 133	4054 ± 351	4174 ± 308

Total cortisone metabolites (Em)	4595 ± 192	4949 ± 311	4701 ± 336
Corticosteroid metabolite ratios (mean ± SEM)			
UFF/UFE (cortisol/cortisone)	0.7 ± 0	0.8 ± 0.1*	0.7 ± 0[§]
(THF+5αTHF)/THE	0.9 ± 0	1.1 ± 0.1	1.5 ± 0.1*
Cortols/cortolones ((α-cortol+ β-cortol)/ (α-cortolone+ β-cortolone))	0.4 ± 0	0.4 ± 0	0.5 ± 0*[§]
(11OH-androst + 11OH-etio)/11oxo-etio	2.9 ± 0.1	5.7 ± 0.8*	9.3 ± 0.8*[§]
5αTHF/THF	0.9 ± 0	1.1 ± 0.1	1.4 ± 0.1*[§]
Androsterone/Etiocholanolone	1.2 ± 0.1	1.8 ± 0.2*	2.5 ± 0.2*

3.3.3 GMLVQ analysis of the urinary steroid metabolome can distinguish early from advanced fibrosis

3.3.3.1 Using GMLVQ to distinguish controls from those with NAFLD

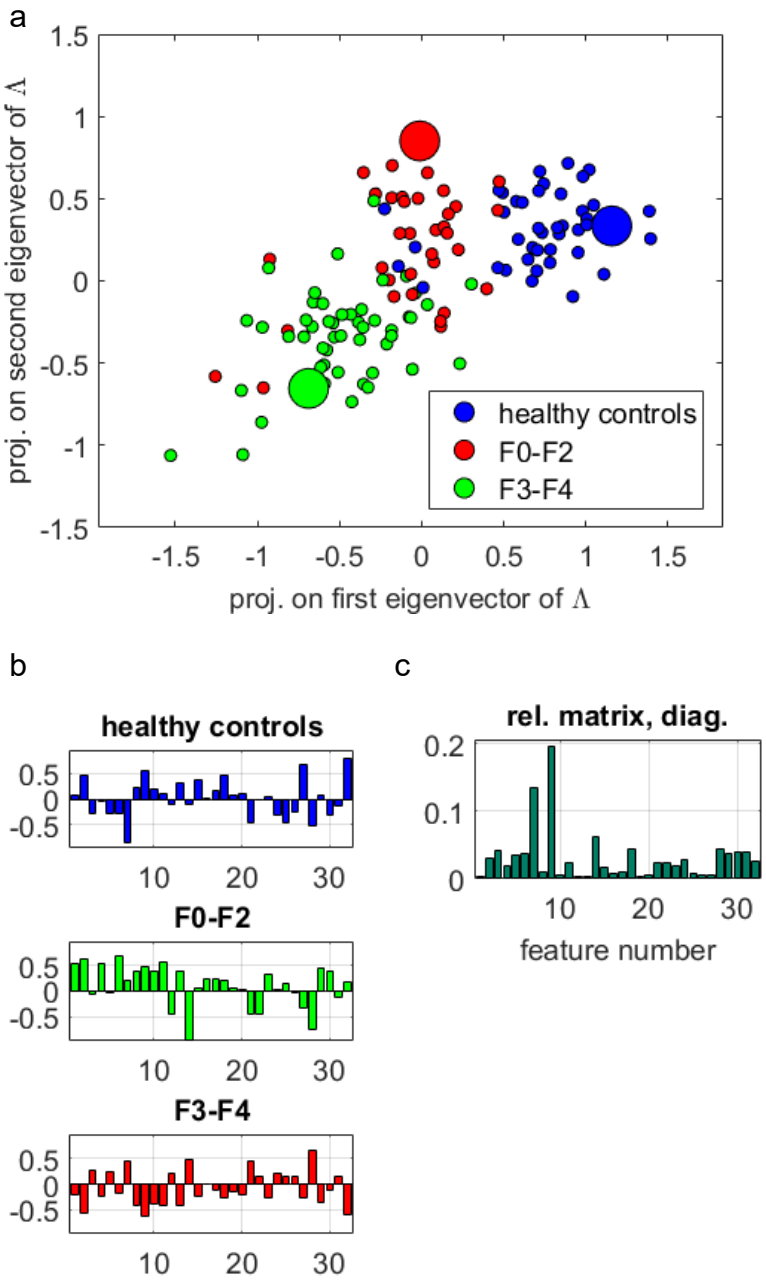
Analysis of data using individual steroid metabolites and ratios demonstrated significant overlap across all groups and was therefore of limited value in being able to correctly determine NAFLD disease stage. Furthermore, manual interpretation and assimilation of data arising from 32 variables is not possible. Accordingly, a global approach was adopted using computational machine learning techniques, in particular GMLVQ mathematical analysis in collaboration with experts in this field from the University of Groningen.

Figure 3.3 shows an initial approach to separate the three broad classes –controls, early fibrosis and advanced fibrosis – from each other and is thus a three class classification paradigm. GMLVQ was used to analyse all 32 urinary steroids and metabolites (Figure 3.3a) in tandem based on the generation of prototype steroid profiles (Figure 3.3b) and an

associated relevance matrix which indicated the importance of individual steroids to the GMLVQ classifier (Figure 3.3c).

Figure 3.9: GMLVQ analysis of patients with NAFLD separated by disease stage (early fibrosis: F0-F2 or advanced fibrosis (F3-F4) compared to controls

Numerical values are given for each individual steroid metabolite (see Table 3.1). Two-dimensional visualization of steroid data obtained by projection of the z-score transformed and log-scaled excretion values onto the first and second eigenvector of the relevance matrix to illustrate discrete separation of disease stage by GMLVQ (a). Prototypical representatives of disease stages illustrating the relative importance of individual steroids using z-score transformed log-scaled steroid excretion values (b). Diagonal elements of the relevance matrix (normalized to sum 1), indicating the importance of individual steroids in the GMLVQ classifier across all disease stages including controls (c).



Having proven the principle that GMLVQ was able to distinguish between the three classes, the research problems were refined to focus on a binary approach to disease classification. In this regard, GMLVQ was able to accurately distinguish between controls and any stage NAFLD (F0-F4) (Table 3.4). Furthermore, as the GMLVQ classifier system is able to incorporate any number of features (dimensions), GMLVQ performance was able to be enhanced by the inclusion of both age and body mass index (BMI) into the model (termed GMLVQ*) (Table 3.4).

Table 3.10: Comparison of GMLVQ analysis of urinary steroid metabolites vs. serum assessments using Fib-4 and NAFLD fibrosis scores

Analysis of samples corrected for urinary creatinine.

	AUC ROC (95% confidence intervals)					
Clinical comparison	**NAFLD Fibrosis score**	**Fib-4**	**GMLVQ (32 steroids)**	**GMLVQ* (32 steroids, age, BMI)**	**GMLVQ-10 (top 10 steroid metabolites)**	**GMLVQ-10* (top 10 steroid metabolites, age, BMI)**
F0-F2 vs. F3-F4	0.87 (0.86-0.88)	0.91 (0.89-0.92)	0.89 (0.87-0.90)	0.92 (0.91-0.94)	0.87 (0.85-0.88)	0.92 (0.91-0.94)
F0-F3 vs. F4	0.87 (0.86-0.88)	0.84 (0.83-0.85)	0.87 (0.85-0.89)	0.92 (0.91-0.94)	0.85 (0.83-0.87)	0.90 (0.89-0.92)
Controls vs. F0-F4			0.93 (0.92-0.94)	0.94 (0.92-0.95)	0.92 (0.91-0.93)	0.94 (0.93-0.96)
Controls vs. F3-F4			0.99 (0.98-0.99)	0.99 (0.98-0.99)	0.98 (0.97-0.98)	0.98 (0.98-0.99)
Controls vs. F4			1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (0.99-1.00)

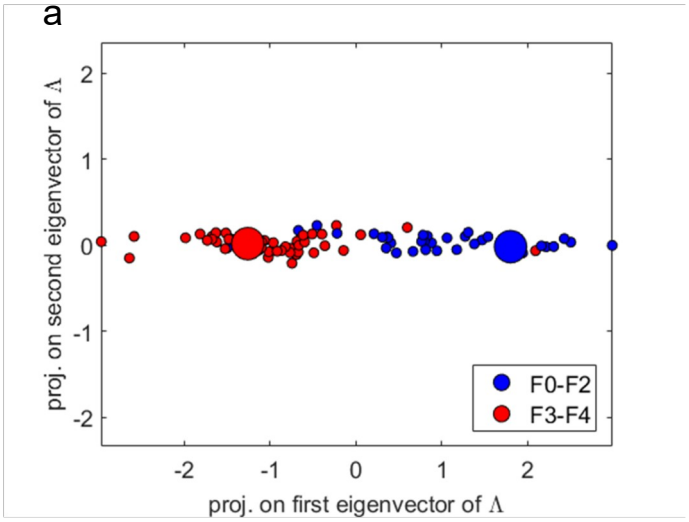
3.3.3.2 Using GMLVQ to risk stratify those with established NAFLD

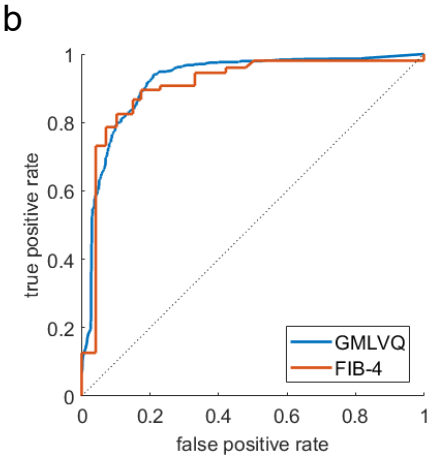
In order to risk stratify those at greatest risk of disease progression and complications in subjects with established NAFLD, GMLVQ was used to classify those who had early (F0-2) from those who had advanced (F3-4) fibrosis. 2D representative plots were produced as shown in Figure 3.4a which demonstrated good separation. Corresponding area under the curve (AUC) analysis of the receiver operating characteristics (ROC) curves suggested that urinary steroid GMLVQ and GMLVQ* performed as well as the established non-invasive serum marker algorithm, Fib-4 (Figure 3.4b, Table 3.4).

Patients with liver cirrhosis (F4) have a greater risk of developing hepatocellular carcinoma and hepatic decompensation than those without cirrhosis (F0-F3) and therefore require active monitoring and surveillance. Both GMLVQ and GMLVQ* were able to identify those patients with NAFLD cirrhosis (F0-3 *vs.* F4) and out-performed non-invasive serological assessments including NAFLD fibrosis score and Fib-4 (Figure 3.4c and 3.3d, Table 3.4).

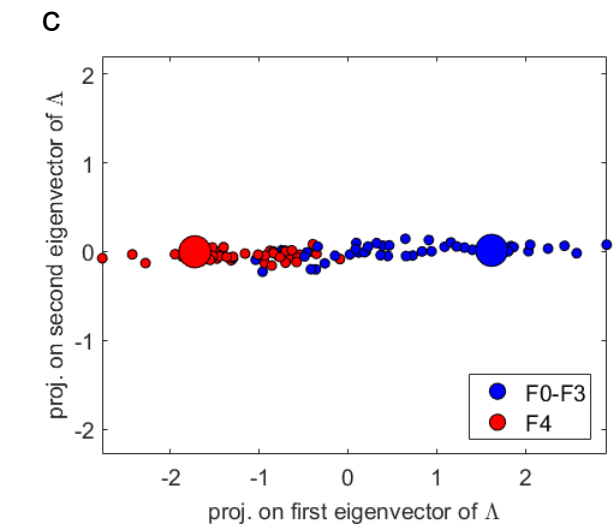
Figure 3.10: GMLVQ analysis permits very good separation between early and advanced fibrosis (F0-2 vs. F3-4) in patients with NAFLD and very good separation between non-cirrhotic and cirrhotic patients with NAFLD

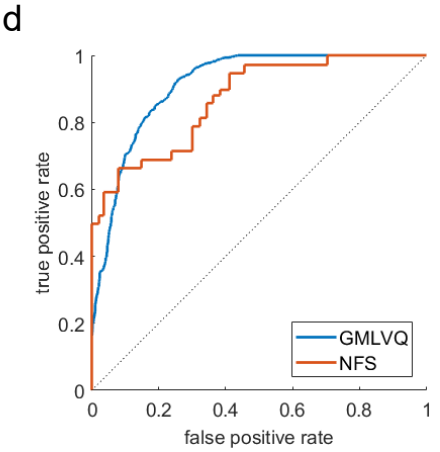
(a) GMLVQ is able to distinguish between early and advanced fibrosis. (b) GMLVQ performance distinguishing between early and advanced fibrosis in comparison to FIB-4. (c) The performance of GMLVQ to identify patients with cirrhosis (F0-3 vs. F4) is also very good. (d) GMLVQ demonstrates significant improvement in diagnostic ability when compared to NAFLD fibrosis score in identifying patients with cirrhotic NAFLD (F0-3 vs. F4).





ROC AUC
GMLVQ*: 0.92 (0.91 - 0.94)
FIB-4: 0.91 (0.89 - 0.92)





ROC AUC
GMLVQ*: 0.92 (0.91 - 0.94)
NFS: 0.87 (0.86 - 0.88)

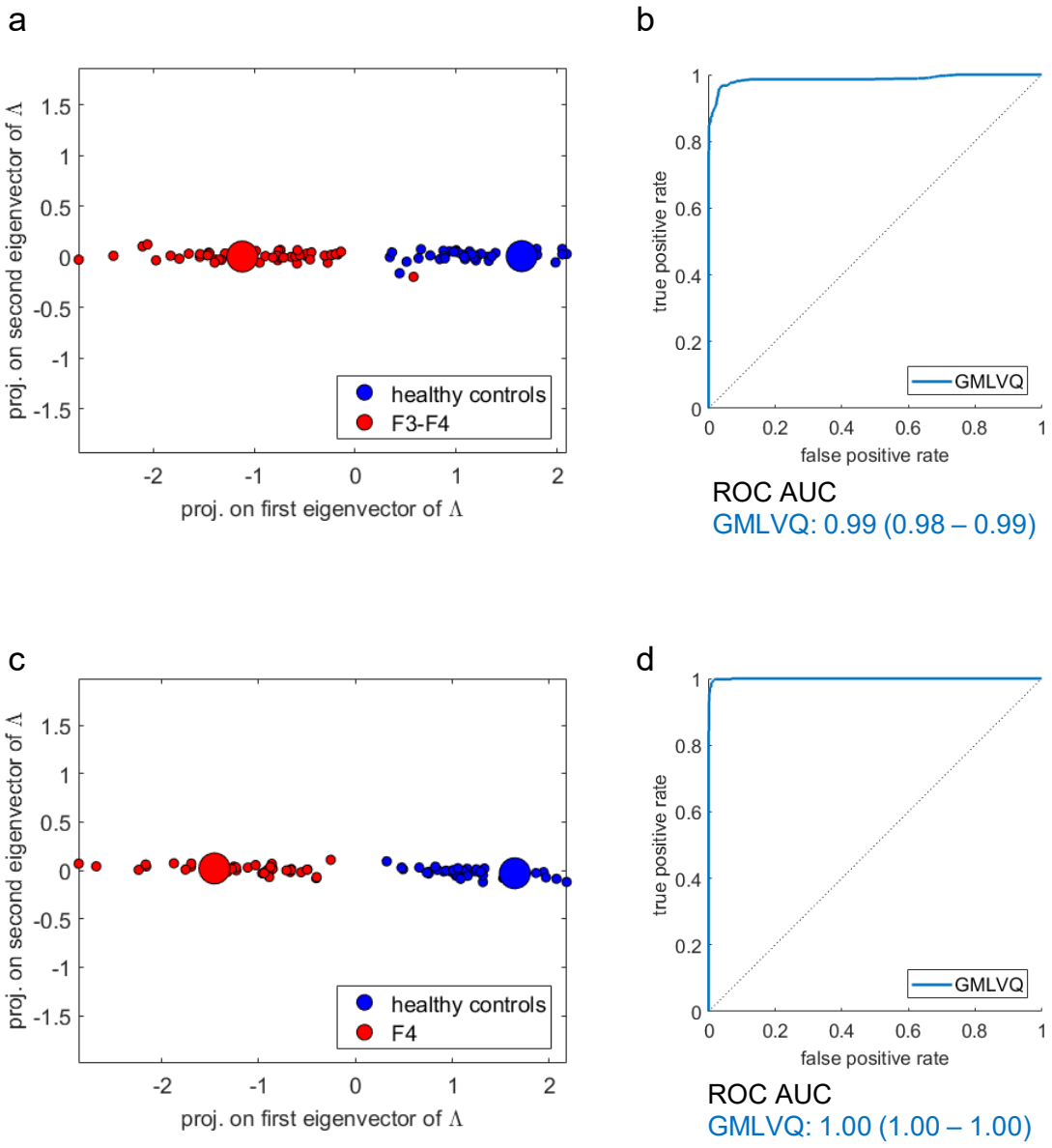
3.3.3.3 GMLVQ as a screening tool has excellent potential to identify people with advanced NAFLD in the general population.

Studies have suggested a high prevalence of undiagnosed advanced NALFD in the general population (302,303), and whilst screening is not currently advocated, identification of

advanced fibrosis and cirrhosis would significantly alter patient management. Both GMLVQ and GMLVQ* demonstrated excellent separation and diagnostic ability in identifying patients with advanced NAFLD from controls (Figure 3.5a and b). When used to identify those patients with liver cirrhosis, there was perfect separation with AUC ROC=1.0 (1.00-1.00, 95% confidence intervals) (Figure 3.5c and d) (Table 3.4).

Figure 3.11: GMLVQ demonstrates excellent separation of controls from those with advanced fibrosis and controls from those with NAFLD cirrhosis highlighting its potential utility as a screening tool to identify individuals with advanced NAFLD fibrosis within the general population

There was excellent separation between controls and those with advanced NAFLD fibrosis (a) with the corresponding ROC AUC analysis (b). The performance of GMLVQ to identify patients with NAFLD cirrhosis in the general population (control vs. F4) was excellent with perfect separation (c and d).*



3.3.3.4 GMVLQ can be refined to include only 10 urinary steroid metabolites without significant loss in diagnostic performance

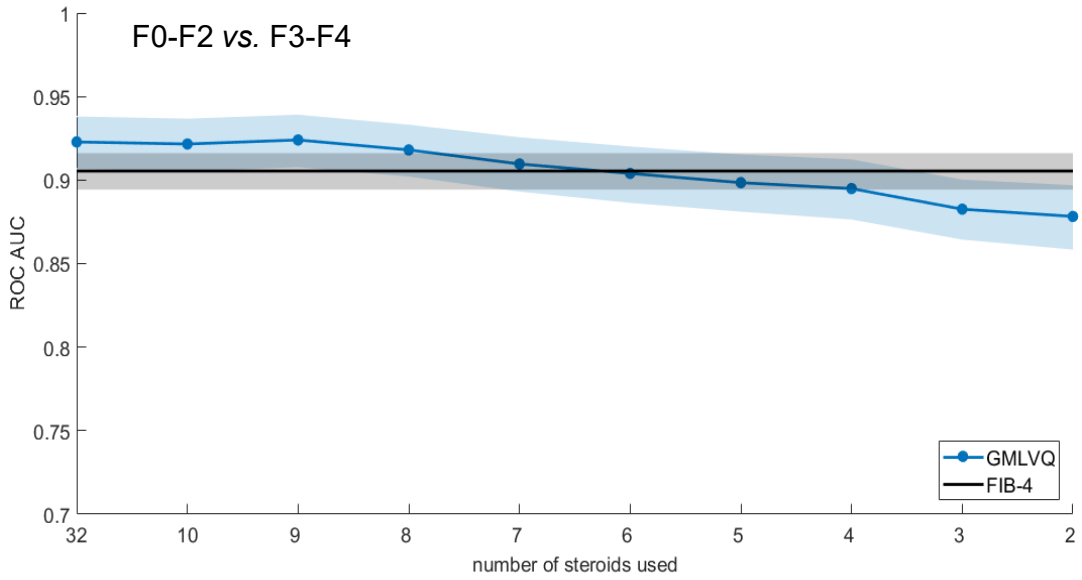
A further GMLVQ analysis was performed with sequential removal of the least discriminatory steroid metabolites. GMLVQ analysis was then compared against the best performing non-invasive serum markers (Fib-4 for F0-2 *vs*. F3-4 and NAFLD fibrosis score for F0-3 *vs*. F4). Refining the model from 32 metabolites to 10 (GMLVQ-10) did not result in any loss of

diagnostic performance and GMLVQ analysis incorporating age and BMI using 10 steroid metabolites (GMLVQ-10*) still out-performed FIB-4 (F0-2 *vs*. F3-4) and NAFLD fibrosis score (F0-3 vs. F4) (Figures 3.6a and b respectively) (Table 3.4). The 10 most discriminatory steroids that had the most impact in distinguishing each of the clinical comparisons (F0-2 *vs.* F3-4; F0-3 *vs.* F4; Control *vs.* F3-4; Control *vs.* F4) are shown in Table 3.5.

Figure 3.12: GMVLQ can be refined to include only 10 urinary steroid metabolites without significant loss in diagnostic performance

The ability of GMLVQ and GMLVQ to identify advanced NAFLD fibrosis (F3-4) (a) and cirrhosis (F4) (b) can be refined to a panel of approximately 10 specific steroid metabolites (GMLVQ-10*) without significant reduction in diagnostic performance.*

a


F0-F2 *vs.* F3-F4
ROC AUC
1
0.95
0.9
0.85
0.8
0.75
0.7
32
10
9
8
7
6
5
4
3
2
number of steroids used
GMLVQ
FIB-4

b

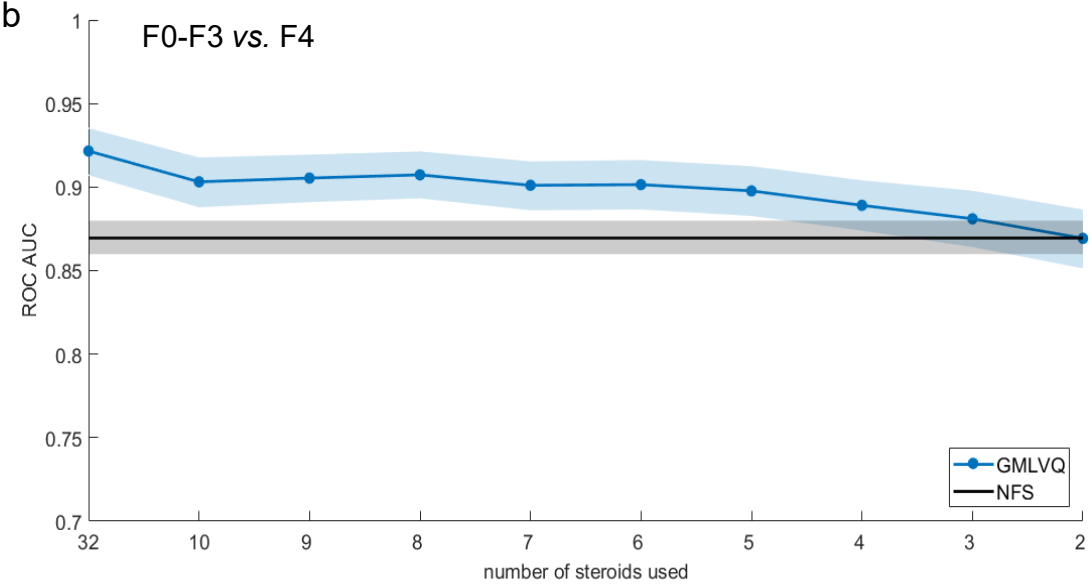

F0-F3 *vs.* F4
ROC AUC
1
0.95
0.9
0.85
0.8
0.75
0.7
32
10
9
8
7
6
5
4
3
2
number of steroids used
GMLVQ
NFS

Table 3.11: GMLVQ analysis identifies the 10 most discriminatory steroid metabolites for distinguishing clinically relevant stages of NAFLD

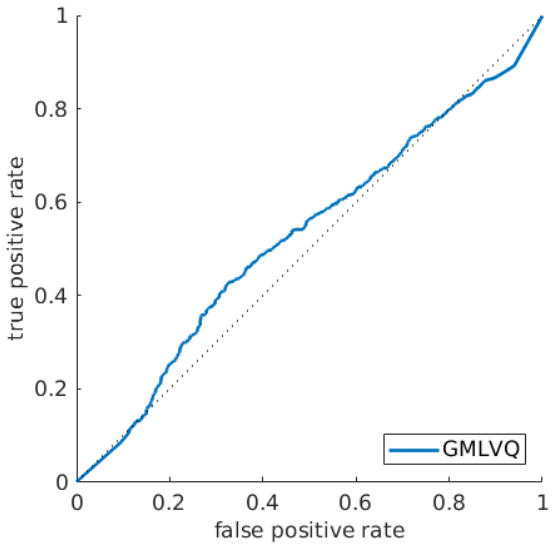
Steroids highlighted in bold are common to all clinical comparisons.

	NAFLD stage comparison			
Discriminatory ranking	F0-2 *vs.* F3-4	F0-3 *vs.* F4	Healthy control *vs.* F3-4	Healthy control *vs.* F4
1	**Etiocholanolone**	**Etiocholanolone**	**5α-tetrahydro-11-dehydrocorticosterone**	**5α-tetrahydro-11-dehydrocorticosterone**
2	Dehydroepiandrosterone	Tetrahydrocorticosterone	11-oxoetiocholanolone	11-oxoetiocholanolone
3	**5α-tetrahydro-11-dehydrocorticosterone**	**5α-tetrahydro-11-dehydrocorticosterone**	**Etiocholanolone**	**Etiocholanolone**
4	Androstendione	Tetrahydro-11 deoxycorticosterone	Cortisone	Cortisone
5	**5α-tetrahydrocorticosterone**	Dehydroepiandrosterone	Pregnenediol	Tetrahydro-11 deoxycorticosterone
6	**Pregnenetriol**	Androstendione	**Pregnanetriol**	Pregnenediol
7	Tetrahydro-11 deoxycorticosterone	Tetrahydrocortisone	Tetrahydro-11 deoxycorticosterone	**Pregnanetriol**
8	Tetrahydroaldosterone	Tetrahydrocortisol	11β-hydroxyetiocholanolone	Tetrahydrocorticosterone
9	Cortisone	**Pregnenetriol**	Pregnanediol	Pregnanediol
10	11-oxoetiocholanolone	**5α-tetrahydrocorticosterone**	**5α-tetrahydrocorticosterone**	**5α-tetrahydrocorticosterone**

3.3.3.5 Additional GMLVQ analyses

Additional GMLVQ analyses were performed with an aim to separating data by gender and NAS score as well as comparing urinary steroid metabolites uncorrected for urinary creatinine. No impact of gender (data not shown) nor NASH (Figure 3.7) was found.

Figure 3.13: GMLVQ analysis was unable to distinguishing between NASH (NAS>4) and non-NASH in those with established NAFLD



ROC AUC
GMLVQ: 0.56 (0.53 – 0.59)

3.3.3.6 Comparison of GMLVQ data using urine steroid metabolites corrected for urinary creatinine or uncorrected for urinary creatinine

AUC ROC analysis was similar using data from samples where uncorrected steroid metabolite levels, which were expressed as µg steroid /1000 mL urine (Table 3.6). GMLVQ analysis both with and without age and BMI added to model performed equivalently to Fib-4 in risk stratifying early from advanced fibrosis. The GMLVQ model incorporating age and

BMI outperformed both Fib-4 and NFS in distinguishing non-cirrhotic NAFLD from cirrhotic NAFLD. GMLVQ also performed very well in separating controls from those with any stage of NAFLD fibrosis and controls from those with advanced fibrosis.

Table 3.12: Performance of urinary steroid metabolites uncorrected for urinary creatinine analysed by GMLVQ

Comparison of GMLVQ and GMLVQ analysis of urinary steroid metabolites vs. serum assessments using Fib-4 and NAFLD fibrosis scores using urinary steroid metabolites uncorrected for urinary creatinine and quantified as µg/1000mL urine.*

	AUC ROC (95% confidence intervals)					
Clinical comparison	**NAFLD Fibrosis score**	**Fib-4**	**GMLVQ (32 steroids)**	**GMLVQ* (32 steroids, age, BMI)**	**GMLVQ-10 (top 10 steroid metabolites)**	**GMLVQ-10* (top 10 steroid metabolites, age, BMI)**
F0-F2 vs. F3-F4	0.87 (0.86-0.88)	0.91 (0.89-0.92)	0.87 (0.85-0.89)	0.92 (0.91-0.94)	0.87 (0.86-0.89)	0.92 (0.90-0.94)
F0-F3 vs. F4	0.87 (0.86-0.88)	0.84 (0.83-0.85)	0.85 (0.83-0.87)	0.90 (0.89-0.91)	0.85 (0.83-0.87)	0.89 (0.87-0.91)
Controls vs. F0-F4			0.94 (0.93-0.95)	0.94 (0.93-0.95)	0.96 (0.95-0.97)	0.96 (0.95-0.96)
Controls vs. F3-F4			0.99 (0.99-1.00)	0.99 (0.98-0.99)	1.00 (0.99-1.00)	0.99 (0.99-1.00)
Controls vs. F4			1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)

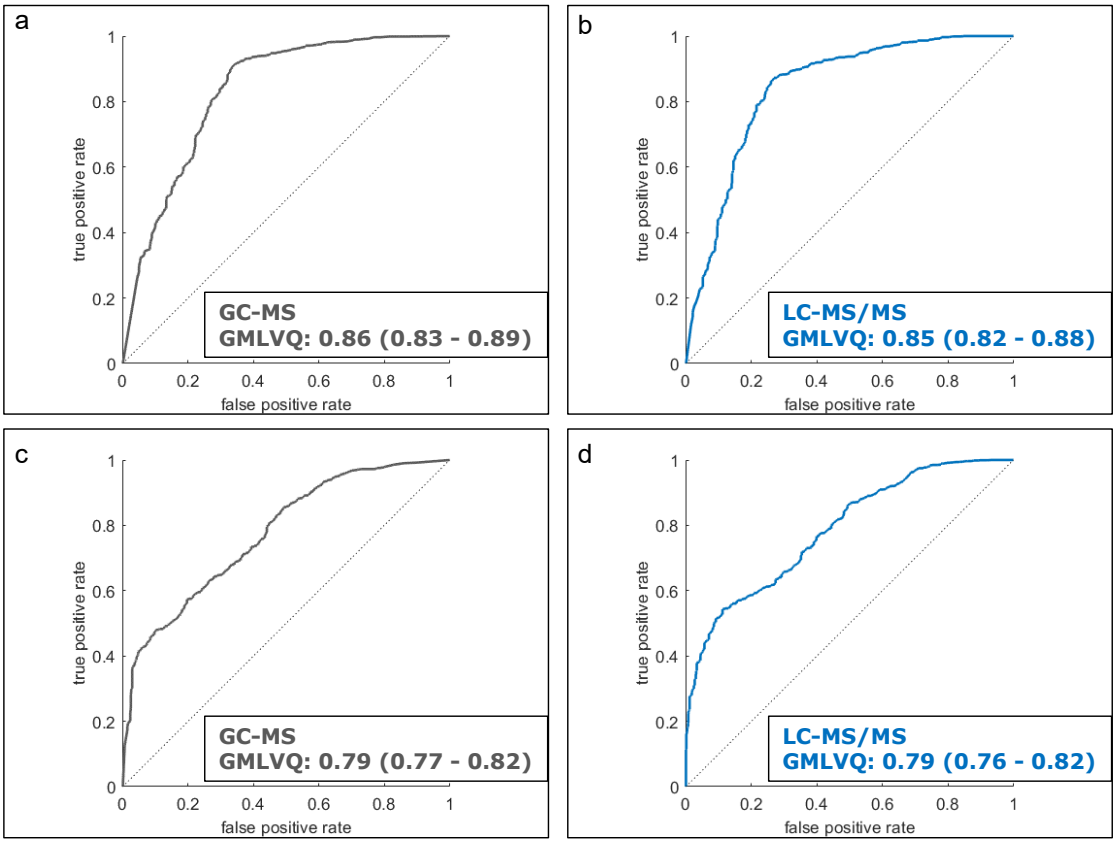
3.3.3.7 Using liquid chromatography Comparison of GMLVQ data using urine steroid metabolites corrected for urinary creatinine or uncorrected for urinary creatinine

GC-MS is time-consuming, labour intensive and expensive which may limit any potential future clinical utility. Consequently, a subset of 77 urine samples from subjects with established NAFLD (F0-F4) were analysed in collaboration with colleagues at the University of Birmingham who have developed a high-throughput liquid chromatography-tandem mass spectrometry platform to analyse urinary steroids. LC-MS/MS is faster and significantly less expensive than GC-MS. Data from a subset of 26 urinary steroid metabolites derived from GC-MS were compared with the same set derived from LC-MS/MS.

When compared with GC-MS, LC-MS/MS performance was equivalent in being able to distinguish early fibrosis from advanced fibrosis as well as being able to distinguish between non-cirrhotic and cirrhotic NAFLD (Figure 3.8).

Figure 3.14: Comparison of GC-MS and LC-MS/MS urinary steroid data using GMLVQ

Using GMLVQ to distinguish early fibrosis (F0-F2) from advanced fibrosis (F3-F4) (a) & (b), and predicting cirrhosis in patients with NAFLD (F0-F3 vs F4) (c) & (d), demonstrates equivalent performance when comparing methods.



3.4 Discussion

The relationship between NAFLD disease stage and clinical outcome is well established (3,36). If appropriate management strategies are to be implemented, for example, via a metabolic hepatology clinic approach as described in Chapter 2, improved investigative tools that do not carry the associated risks and limitations of liver biopsy and which can be performed in primary care settings, are needed. There is a therefore a pressing need for the development of sensitive and specific non-invasive markers to of stage NAFLD, fuelled, at least in part, by the poor performance of simple routine liver biochemistry (292) which remains the most widely employed screen used by healthcare professionals.

My research demonstrates that analysis of the urinary steroid metabolome combined with machine learning computational techniques such as GMLVQ has the potential to fulfil this need. Firstly, it was able to screen for and accurately identify patients with NAFLD.

Furthermore, this approach was able to accurately classify those with the most advanced stages of disease including liver cirrhosis, in subjects with established NAFLD. If validated, this ability to both screen for and accurately risk stratify patients using a non-invasive technique holds high promise for future use in clinical practice.

A range serological tests, algorithms and imaging modalities that perform reasonably well in their ability to identify disease severity and stage are described in Chapter 1. Imaging modalities including magnetic resonance spectroscopy (MRS) and imaging (MRI) provide accurate assessment of hepatic triglyceride content (304). Identifying inflammation within the liver is however more challenging. Whilst there is some potential from novel imaging platforms and serological tests, for example the measurement of cytokeratin-18 fragments or cathepsin D (305–308), AUC ROC analysis is less impressive than non-invasive biomarkers which stage fibrosis. The number of potential tests that can be used to assess the risk of advanced fibrosis is large. Data from more than 20 different tests, algorithms or imaging platforms have been published (1) and the large number of tests proposed reflects the need for improved performance. ROC AUC performance ranges are broad for many of these tests and although these may sometimes exceed 0.90, the majority of studies suggest values of between 0.8 and 0.9. The potential use of a urinary test is novel. Untargeted urinary metabolic profiling in small numbers of patients has been explored (309–311), but its diagnostic ability has not been interrogated in detail.

As the liver is the main site of steroid hormone metabolism, the research outlined in this chapter was based on the hypothesis that assessment of urinary steroid metabolites can provide a functional assessment of liver function that differs according to NAFLD stage. Previous work has focussed on specific steroid pathways that appear to be dysregulated. For example, there is evidence for increased 11β-HSD1 activity – which was observed in my study – in patients with NASH, and it has been postulated that the resultant increased cortisol generation may serve as an auto-regulatory mechanism to limit local hepatic inflammation (293). In the same study, and contrasting with my data, 5α-reductase activity –

to clear cortisol through the generation of tetrahydrocortisol – was decreased. The reasons for this discrepancy are not clear, although the small number of participants and the lack of detailed histological staging (including fibrosis stage) in previously published studies need to be considered. An additional study has suggested that 5β-reductase activity (which is almost exclusively expressed in the liver), correlates with liver fat content (100).

There is clear biological plausibility in the steroid metabolomic approach. Manipulation of steroid metabolising enzyme activity can impact upon hepatic phenotype. Inhibition of 11β-HSD1 decreases hepatic steatosis (albeit with a modest effect size) (312). Similarly, combined 5α-reductase type 1 and 2 inhibition with Dutasteride increased hepatic triglyceride accumulation as well as driving insulin resistance (103,313). Furthermore, inflammatory stimuli have been shown to regulate the expression and activity of steroid metabolising enzymes (314,315).

The urinary steroid metabolome analysis using GMLVQ has also previously been used to help differentiate benign from malignant adrenal tumours (316) and as such there is a good evidence base and framework for its use in the clinical setting with established methods available and published. Its use in the context of NAFLD is however entirely novel and could potentially be very powerful. Data from this study (AUC ROC >0.9) would suggest excellent potential for GMLVQ analysis of urinary steroids as a strategy for accurate identification and risk stratification of those patients with advanced fibrosis. Furthermore, it performs as an almost perfect test in the identification of patients with advanced fibrosis and cirrhosis when compared against a control population. This may be relevant for identifying patients within the general population that have the most advanced liver disease who are at high risk of cardiovascular and hepatic co-morbidities and complications. This will help health planners and clinicians target management accordingly, for example, primary care management pathways could be established those at lose risk and pathways for referral to secondary care could be established for those at high risk. In addition, it may help with ongoing follow-up

and monitoring in both primary and secondary care and guide discharge back to primary care when risk has been lowered following clinical intervention in suitable patients.

Estimates suggest that prevalence of compensated cirrhosis is likely to rise in the general population by more than 150 % in some countries over the next 10-15 years and therefore identification of these patients is of huge clinical significance (317).

This study is not without its limitations. The data presented is derived from a relatively small cohort of patients, although it should be noted that biomarker exploration in the context of NAFLD has typically been undertaken in cohorts of this size (120). This was also a retrospective study with subjects recruited and analysed from various centres. Consequently, the group sizes are not uniform across the different groups, the cohort presented is skewed towards more advanced fibrotic disease rather than reflecting the distribution of NAFLD severity in the general population and it was not possible to compare individual fibrosis groups separately due to limited sample size.

There was similarly an imbalance in the prevalence of diabetes across experimental groups. It should be noted however, as outlined in chapter 1, that there is a clear but complex relationship between the presence of advanced NAFLD fibrosis and type 2 diabetes and, as such, any biomarker computational model should be developed in acknowledgement of this fact. In recognition of this, during development and testing of the GMLVQ model, the presence of T2DM (yes/no) as well as HbA1c numerical data, were incorporated as potential predictors into the GMLVQ model in separate iterations in a similar manner to age and BMI. In all such iterations, these diabetes predictors were revealed as having relatively less importance in the relevance matrix of top discriminators between groups and importantly, did not improve on ROC AUC values already presented in this chapter (data not shown). As this test has good potential for future use as a screening tool potentially in community healthcare environments, not having to reply on serological data as this did not improve performance is a significant added advantage which will make its use in a screening context simpler, faster and cheaper.

It is also important to note that it was not possible to provide a uniform or standard 'health screening' panel for all groups presented in this chapter. This is particularly relevant for the control group who did not undergo liver biopsy and where not all serological and clinical parameters were available, for example to calculate non-invasive scores to asses for advanced fibrosis. Previous studies have clearly shown the close association between BMI and obesity with NAFLD, including the Dionysis study, which found the prevalence of fatty liver of any severity with or without fibrosis in the obese population to be >75 % and possibly as high as 95 % (318,319). Given the fact that the mean BMI of controls was in the obese range, it is possible that a significant number of controls may have in fact had some degree of NAFLD. Consequently, there is clearly an important though exciting need to validate the findings from this study in larger cohorts with detailed histological staging of disease and comprehensive clinical characterisation, including in control subjects.

Whilst the 'gold-standard' for urinary steroid metabolome analysis is usually a timed 24-hour collection, in this study I have presented spot urine comparisons between groups. Going forward, a spot urine test would have far more practical applicability than a 24-hour urine collection as a non-invasive assessment. Furthermore, as a screening tool, a simple spot urine sample could be collected immediately, for example at a routine health check, and potentially without the need for any further investigations such as blood tests or imaging. The current gold standard methodology of GC-MS is also time consuming and labour intensive. However, the subgroup analysis employing LC-MS/MS has shown that it is possible to use a high-throughput liquid chromatography tandem mass spectrometry approach which offers significant savings both in terms of cost and time and would thus increase appeal for future clinical use.

3.5 Conclusion

This chapter has outlined an entirely novel approach to the diagnosis and staging of NAFLD using measurement of urinary steroid metabolites. Furthermore, coupling this data with machine learning algorithms has potentially allowed for the generation of a novel meaningful biomarker tool. This could have excellent future clinical utility in the assessment and staging of NAFLD, both in secondary care, for example if used in a metabolic hepatology clinic as described in Chapter 2, and in the broader general population potentially to screen for both the presence of NALFD and its severity and reduce the need for liver biopsy.

Chapter 4 Novel interventions for the treatment of NALFD – a head to head comparison of GLP-1 analogue therapy and lifestyle intervention

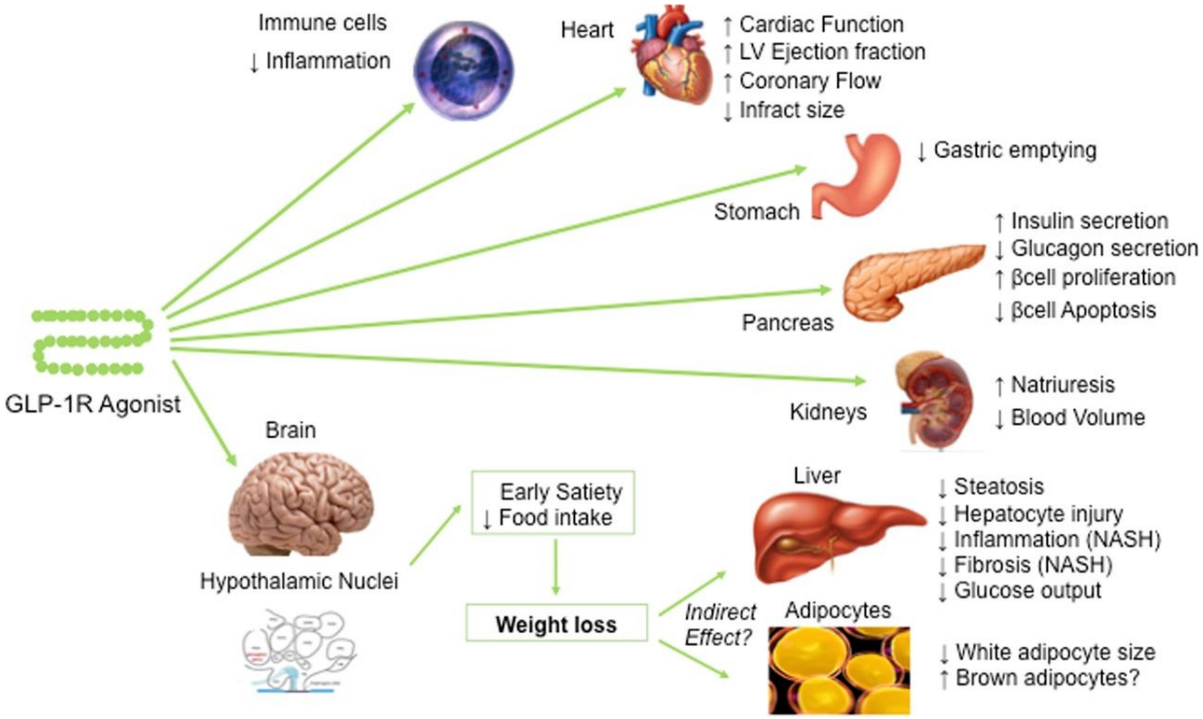
4.1 Introduction

The second theme highlighted from the metabolic hepatology clinic analysis presented in Chapter 2 concerns the approach to management and current therapeutic interventions at the clinician's disposal to tackle the NAFLD epidemic. In particular, there is an urgent need for the first licenced therapy for NAFLD to augment and support the current mainstay of achieving meaningful weight loss through dietary and lifestyle changes.

As described in Chapter 2, 20 % of patients with diabetes attending the NAFLD clinic were commenced on GLP-1 agonist therapy, which promotes weight loss and is currently licenced in the UK for use in obese patients with poorly controlled diabetes. GLP-1 agonists have a range of metabolic effects both peripherally in the body to reduce appetite and centrally within the brain to promote satiety (See Figure 4.1) which promote weight loss. In addition, they have other beneficial actions including promoting glucose dependent insulin secretion which improves glycaemic control and the down regulation of inflammation (320,321). Furthermore, the cardioprotective features of GLP-1 therapy are now well proven with a number of large-scale studies including the LEADER and SUSTAIN-6 trials demonstrating clear cardiovascular benefit and together strongly suggesting a class effect (322). Coupled with this is the added advantage that GLP-1 therapy is safe and well tolerated in most patients with a good side effect profile with available evidence thus far suggesting no concerns with long term use. The primary side effect is of gastrointestinal disturbance (nausea, flatulence, diarrhoea), which often improves if patients are able to persist with therapy and previous concerns regarding a possible increased pancreatitis risk have been allayed with recent evidence finding no increased risk (323,324).

Figure 4.15: Effects of glucagon-like peptide-1 receptor agonists in humans

Reproduced from Dhir and Cusi (320)



In regards to effects on the liver, the GLP-1 agonist Liraglutide has been shown in studies to be useful in NAFLD with a reduction in liver enzymes (170) and in the seminal LEAN study improved the histological appearance of the liver in patients with biopsy proven NASH both with and without type 2 diabetes (171), with studies using other GLP-1 agonists demonstrating similar findings (325).

What remains hitherto unclear is whether this benefit is solely due to the weight loss action alone or whether there exist any direct GLP-1 effects on the liver. The GLP-1 receptor is expressed widely in pancreatic and other tissues including the brain, lungs, gut and muscle (320) though, its expression in the liver is still under debate. Some studies have reported the GLP-1 receptor to be present on human hepatocytes (326) and others have suggested a specific GLP-1 effect on the liver acting to decrease lipid synthesis and accumulation (182),

though presence of the receptor has not been found in other studies (183,327). As such, an important clinical question remains. Liraglutide's putative direct action on the liver is still under considerable debate, though its ability to promote weight loss is well known. It is possible that the beneficial effect on NASH observed in the LEAN study may have been mediated exclusively via weight loss. Nevertheless, the magnitude of the histological improvement of the liver, however, probably exceeded that which might have been anticipated from weight loss alone.

To seek to answer this question on any weight independent effects of Liraglutide in NASH, I established the 'Lifestyle and Liraglutide in NASH' (LiLi) clinical trial described in this chapter, which contributes to the evidence base that may in due course inform medicines licensing agencies to licence GLP-1 agonist therapy as one the first licenced treatments for NAFLD. This study was registered with the MHRA and the HRA and set up as a clinical trial of an investigational medical product (CTIMP) with the study sponsor being the University of Oxford. It provides a direct comparison between lifestyle induced weight loss when matched to Liraglutide therapy promoting equivalent weight loss and thus allows investigation of the weight independent effects of GLP-1 therapy in subjects with NASH.

The hypothesis for this study was that clinical improvement in subjects with NASH taking a GLP-1 agonist exceeds that which may be attributed to weight loss alone.

4.2 Methods

4.2.1 Study description

This was a single centre, open label, randomized phase IIa clinical trial to evaluate the effects of the GLP-1 agonist Liraglutide compared to lifestyle induced weight loss on NASH over a period of 24 weeks. The clinical study protocol received full ethical approval from the East of England - Cambridgeshire and Hertfordshire Research Ethics Committee (REC ref.

16/EE/0403), and the NHS Health Research Authority (HRA). The trial was sponsored and monitored by the Clinical Trials and Research Governance (*CTRG*) research support unit at University of Oxford. The trial was registered as a CTIMP with the UK MHRA and was also registered with European Clinical Trials Database (*EudraCT Number 2016-002045-36).* All subjects provided informed written consent prior to participation.

4.2.2 Eligibility criteria

Individuals with a diagnosis of NASH as defined in the eligibility criteria outlined below and without type 2 diabetes (to remove any confounding effects of improvement in glycaemic control), were recruited from potential subjects identified and invited from clinical databases at OUH NHS Foundation Trust including the metabolic hepatology clinic described in Chapter 2.

Inclusion criteria:

- Non-diabetic (HbA1c <48 mmol/mol)
- BMI 25-50 kg/m^2
- Age 18-75 years
- Diagnosis of NAFLD (hepatic steatosis on imaging or histology)
- ALT ≥1.5 ULN & <10 ULN on 2 occasions >7 days apart and / or
- Historical liver biopsy showing NASH and/or ≥F1 fibrosis or NFS ≥ -1.455 OR Fib-4 ≥ 1.3 OR Fibroscan ≥8 kPa.
- Non-pregnant, not planning pregnancy
- BP<160/100mmHg – stable anti-hypertensive therapy for 6 months
- No weight change >10 % within the preceding 3 months
- Normal renal function
- No contraindications to Lifestyle or Liraglutide

Exclusion criteria:

- Age <18 or >75 years
- Body mass index <25 or >50 kg/m^2
- A diagnosis of diabetes (type 1 or type 2)
- Use of anti-diabetic or weight loss medications or a GLP-1 agonist such as Liraglutide
- Contra-indication to Liraglutide
- A blood haemoglobin <120 mg/dL
- History of alcoholism or a greater than recommended alcohol intake (Recommendations >21 units on average per week for men and >14 units on average per week for women)
- Pregnant or nursing mothers
- History of severe claustrophobia
- Presence of metallic implants, pacemaker that are contra-indications to MRI scanning
- Haemorrhagic disorders
- Anti-coagulant treatment
- Other co-morbidities that in the eyes of the investigators may affect data collection
- Any medical condition in the opinion of the investigators that might impact upon safety or validity of the results

4.2.3 Clinical protocol

Volunteers that met the inclusion criteria at screening and were willing to partake in the study were randomised to one of two 12-week weight loss interventions; a lifestyle intervention

provided by the commercial weight management provider Slimming World or to a Liraglutide drug treatment arm.

These interventions and timescales were chosen to achieve an approximate 5 % weight loss at 12 weeks. This was based on the knowledge that a wide range of trials have been published which show that commercial lifestyle and weight loss programmes are successful at assisting participants in achieving weight loss and that 5 % weight loss is generally accepted as both an achievable and significant amount to bring about health benefits. Both Jebb *et al.* (151) and Jolly *et al.* (328) reported large trials investigating weight loss treatments and found that commercial weight loss programmes achieved a 5 % weight reduction at 12 weeks. Similarly, previous studies have shown that a dose of 1.8mg of Liraglutide daily is able to decrease weight by approximately 5 % (182,329).

All participants in the lifestyle intervention arm attended weekly sessions with Slimming World and followed standard Slimming World printed, online, group and face-to-face guidance and the advice of their trainer. All participants on the Liraglutide arm were taught to self-inject the drug subcutaneously and commenced on a starting dose of 0.6 mg daily which was up-titrated weekly by 0.6 mg to a final dose of 1.8mg daily as per usual prescribing guidelines. Participants kept a diary record of daily drug administration. Those who suffered side effects and were not able to tolerate 1.8mg were reduced to the lower dose of 1.2 mg and maintained on this lower dose. All participants in the Liraglutide arm were provided general lifestyle advice as per routine clinical care and were advised not to make significant alterations to their pre-existing diet or lifestyle.

A standardized and validated tool – the International Physical Activity Questionnaire (IPAQ) – along with 7-day pedometer data and food diary – were used at baseline, 12 -weeks and 24-weeks to assess physical activity and dietary intake in all participants to objectively determine any difference between groups during the intervention and follow-up periods.

Figures 4.2 and 4.3 provides a summary of the research protocol and investigations performed at baseline prior to the instigation of weight loss intervention and then repeated at 12-weeks (with additional interim visits for follow-up occurring at weeks 1, 4 and 8). Patients then discontinued the Liraglutide or lifestyle intervention supported as part of the study and a repeat set of investigations to assess legacy effects were performed at 24 weeks.

Figure 4.16: LiLi study: protocol overview

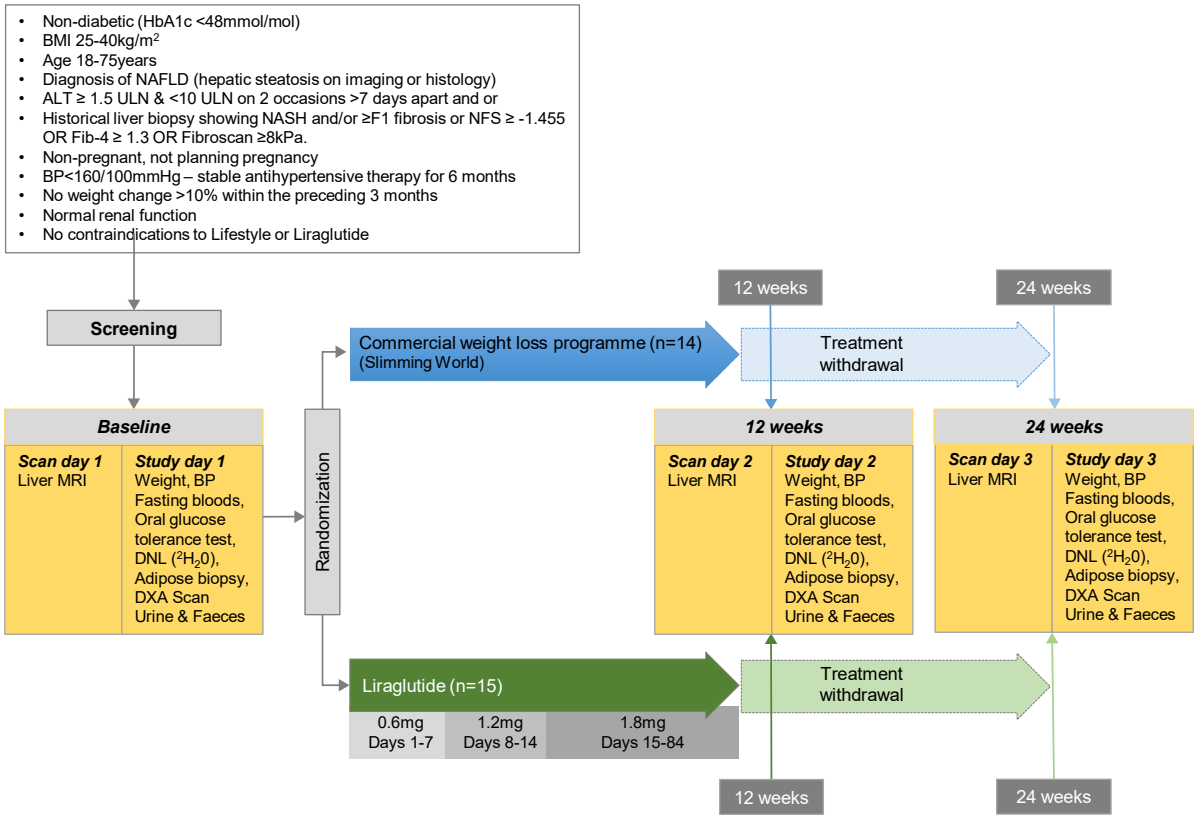
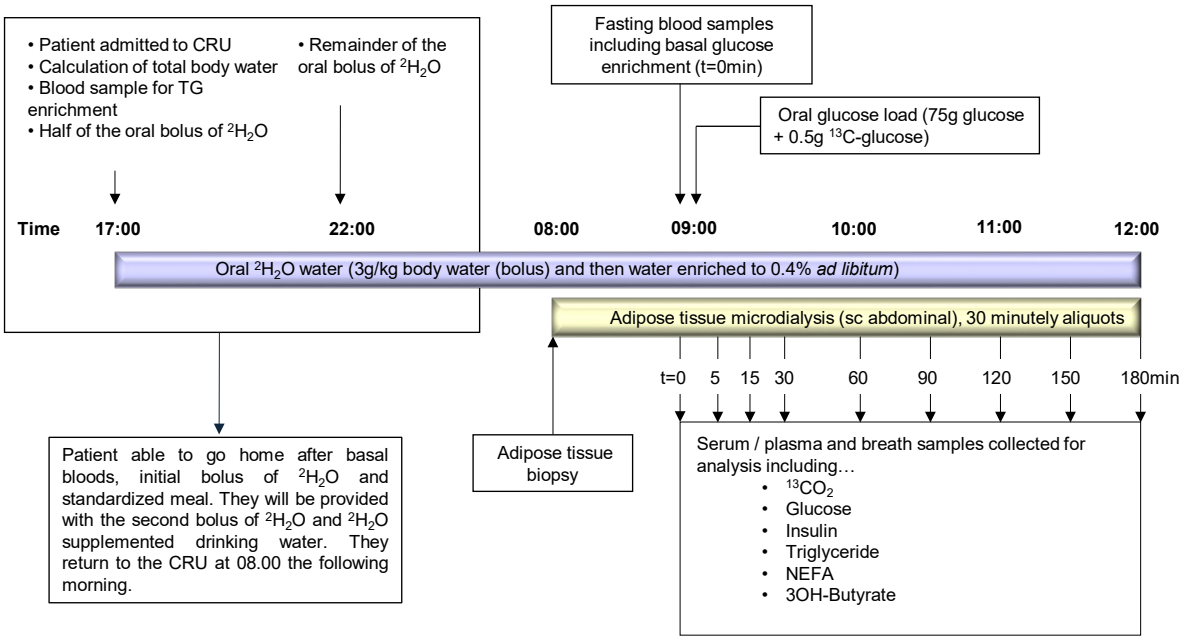


Figure 4.17: LiLi Study: metabolic study day protocol (prolonged oral glucose tolerance test)



Baseline investigations were typically performed over two days and included assessment of hepatic triacylglycerol (TAG) content using magnetic resonance spectroscopy and hepatic fat and inflammation using multiparametric magnetic resonance (cT1) on the first day. This was followed by a metabolic study day (Figure 4.3) performed in the fasted state (except for the consumption of low concentration deuterated (drinking) water – see DNL section), to assess lipid and glucose handling and other metabolic activity within the liver and the body. Baseline investigations (imaging and metabolic assessments) were performed, after which time the study intervention was commenced within 1 week. Interim visits at weeks 1, 4 and 8 allowed for assessment of compliance with the intervention, blood test monitoring and assessment of any adverse drug events where applicable. All investigations were repeated after 12-weeks of treatment, after which all interventions supported through the study were

stopped. All samples collected were processed where applicable and stored at -80 degrees in line with all ethically agreed and regulatory protocols.

4.2.4 Anthropometry and body fat distribution

Anthropometric measures were collected at each visit. Body composition data including total and regional body fat mass and distribution was measured using dual-energy X-ray absorptiometry (DXA) (GE Lunar iDXA machine), which has been previously validated to have good concordance with more detailed imaging modalities in this regard and has been shown to to correlate more strongly with metabolic risk factors than traditional anthropometry including BMI and waist and hip circumference(330). Regions of interest were automatically defined by Encore software (version 14.0; GE Healthcare, Bucks, UK) and have previously been described in detail (331). All DXA measurements were recorded on a single DXA scanner which was calibrated daily using a GE Lunar calibration phantom. Scans were performed after calibration passed quality control by trained staff according to a standard operating procedure.

4.2.5 Physical activity

All participants were provided with an Omron Walking Style 2.1 (HJ321E), 'wear almost anywhere' 3D pedometer (Omron Corporation, Japan), and asked to wear it for 7 days immediately prior to or following the metabolic study day. The pedometer was calibrated as per manufacturer instructions for each participant at baseline and the mean step count per day was calculated at baseline, 12 and 24-weeks. Step counts for any individual day were only included in the 7-day average analysis if the pedometer recorded >1000 steps on that particular day.

All participants were asked to complete the short, 'last 7 days', self-administered format International Physical Activity Questionnaire (IPAQ) which is a validated instrument to

measure physical activity (332,333). Data were summarised and are presented as 'physical activity' (PA) per week to include all of walking, moderate and vigorous exercise or as time spent sitting per day.

4.2.6 Magnetic resonance imaging

All magnetic resonance studies were performed at the University of Oxford Centre for Clinical Magnetic Resonance Research (OCMR) using a 3 Tesla system (Tim Trio, Siemens Healthcare, Germany) by radiographers trained in the relevant scanning techniques. Participants attended for their scans having fasted for at least 4 hours. Participants were positioned supine as default unless large body habitus or patient preference required scanning to be performed in the prone position. All scans for the same participant were performed in the same position.

Hepatic steatosis (liver fat fraction) was quantified using localised cardiac-triggered proton spectroscopy. Steatosis as a percentage of the liver fat content using ^{1}H MRS was measured with and without water suppression in an 8 cm^3 voxel, avoiding vascular and biliary structures (141) in the same region of the liver for each participant at each visit. MRS data was batch analysed offline, using AMARES as implemented by the OXSA toolbox (334) in MATLAB (The Mathworks, MA, USA).

To characterise extracellular water content, a T1 relaxation time map was acquired using the shortened Modified Look Locker Inversion (shMOLLI) recovery sequence taken in a transverse plane of the liver (335). This sequence sampled the T1 recovery curve using single-shot balanced steady state free precession acquisitions. Iron was quantified using a multiple echogradient-echo acquisition which allowed calculation of a $T2^*$ map of the liver in a single plane (slice thickness of 3 mm). To correct for the effect of iron on T1, a shMOLLI sequence was modelled using a Bloch equation simulation for varying extracellular fluid and iron concentrations, and a correction lookup table was generated. This was then used to

remove the effects of elevated iron from the T1 measurements, yielding an 'iron-corrected T1' (cT1) which has been used as surrogate marker of inflammation and fibrosis (17).

LiverMultiScan software, LMS Discover (version 4.1.3) was used to calculate proton density fat fraction (PDFF) to estimate liver fat percentage, to calculate hepatic iron content and to determine cT1 values. This analyses was performed by Perspectum Diagnostics.

Sequence parameters for the imaging study were as follows. For MRS, TR was cardiac-gated and at least 2 s long for water-suppressed spectra and at least 4 s long for non-water-suppressed spectra, TE = 10 ms, and TM = 7 ms. Voxel size was 20 × 20 × 20 mm^3 for both spectroscopy experiments. For shMOLLI: TR = 2.41 - 2.45 ms, bSSFP TE = 1.02 - 1.05 ms, FOV 270–300 × 360–400 mm^2 (depending on patient body size), 24 k-space lines before the central line, 66 total k-space lines, shortest inversion time was either 100 ms or 110 ms, inversion time increment 80 ms. This acquisition was also synchronised to the diastolic phase of the cardiac cycle. For Multi-echo GRE: FA = 6°, TR/TE = 22.2/1.25, 2.46, 3.69, 4.92, 6.15, 7.38, 8.61, 9.84 ms, FOV 302 × 360-400 mm^2, acquisition matrix 128 × 104, phase encoding direction anterior-posterior, BW = 1180 Hz/px, bipolar gradient readout scheme and slice thickness 3 mm.

4.2.7 De novo lipogenesis (DNL): 16:0 enrichment of palmitate from heavy water

To assess DNL and hepatic fatty acid synthesis, participants consumed 2 pre-specified doses of deuterated 'heavy water' (^2H_2O), (CK Gas Ltd) the evening prior to the study day at 1800 and 2200 respectively. The dose (volume) of heavy water consumed at 1800 and 2200 was calculated as follows:

- *Volume of heavy water (ml) = [(total body water (kg) X 3) / 2]*

Additionally, from the first dose of heavy water at 1800 onwards, until the end of the study day visit the next day, participants were provided with and asked to only drink water

enriched with deuterated water at a low concentration of 4 g/l (0.4%) to be drunk *ad libitum* in lieu of any other fluids.

Incorporation of ^{2}H from the $^{2}H_2O$ into TAG-palmitate was used to determine hepatic DNL. Hepatic DNL was measured on the study day, based on the incorporation of ^{2}H in plasma water (Finnigan GasBench-II;ThermoFisher Scientific, UK) and into TAG palmitate using GC-MS (336). DNL was calculated using the following formula:

> fraction = ($\Delta^{2}H$ to ^{1}H ratio in palmitate methyl ester/$\Delta^{2}H$ to ^{1}H ratio in water pool) (34/22), where 34 is the total number of H atoms in palmitate methyl ester and 22 is the number of water molecules incorporated into palmitate via DNL

4.2.8 Fatty acid oxidation

To assess fatty acid oxidation, a modified oral glucose tolerance test (OGTT) incorporating a carbon-13 stable isotope tracer (75g glucose + 0.5g ^{13}C-glucose) was performed. Breath samples were collected at half hourly intervals from baseline throughout the OGTT until 180 min (see Figure 4.3) over the study day and the generation of $^{13}CO_2$ was measured as reflection of global fatty acid oxidation. $^{13}C/^{12}C$ ratios in breath samples and the relative rate of whole-body fatty acid oxidation was calculated as previously described (337). The rate of expiration of $^{13}CO_2$ in breath was calculated by multiplying the estimated VCO_2 (338) by the enrichment of breath CO_2.

4.2.9 Biochemical analysis

Fasting biochemical bloods (full blood count, c-reactive protein (CRP), renal function and liver chemistry and lipid profile) were analysed through the NHS clinical laboratories at Oxford University Hospitals NHS Foundation Trust. Blood glucose was measured using the

HemoCue Glucose 201 DM RT system (HemoCue, Angelholm, Sweden). For other blood parameter analyses over the course of the metabolic study day, whole blood was collected in heparinised syringes (Starstedt, Leicester) and centrifuged for 10 minutes at 825 g 4°C (Eppendorf Centrifuge 5702R, Stevenage, UK) to isolate plasma which was stored at -80°. Serum insulin was measured using a commercially available ELISA assay (Mercodia, Uppsala, Sweden) according to the manufacturer protocol and insulin resistance (HOMA-IR) was calculated using established criteria (339).

4.2.10 Statistical power and analyses

To ensure sufficient statistical power, a sample size of n=25 in each arm was planned to account for a 10 % drop-out rate, incomplete sample collection, accurate matching of weight loss, and sample loss during analysis. It was envisaged that this would allow for a minimum of 20 % reduction in liver fat as measured by MRS imaging, based on weight loss of 5 % at 12 weeks and to detect at least a 25 % change in rate of DNL (power=0.8, α=0.05) based on prior studies (151,182,328).

An interim analysis (n=29) of all who completed the 12-week intervention (primary outcome) of the study (n=14 liraglutide and n=15 lifestyle), identified that both groups were matched for weight loss and that there was a >20 % reduction in liver fat as measured by MRS, which satisfied the envisaged plan to achieve statistical power.

For parameters measured across the OGTT, areas under the curve were calculated by the trapezoid method and have been divided by the relevant time period to give time-averaged values.

Paired t tests or their non-parametric equivalents were used to compare outcomes before and after individual treatments. Unpaired t-tests were used to test across and compare interventions. Where multiple comparisons were made, repeated measures ANOVA

analyses were used to determine the effects of each intervention. The statistical significance level for all results was set as 5%.

4.2.11 Primary outcome

The primary outcome for the study was change in liver fat using MRS performed at baseline, and repeated at 12 weeks to evaluate changes to liver fat.

4.3 Results

4.3.1 Baseline data

33 of 49 volunteers who successfully passed screening enrolled into the study. 31 participants continued on in the study until the end of study day 1 to be randomised to either lifestyle or Liraglutide intervention. 29 participants (n=15 lifestyle, n=14 Liraglutide) completed the 12-week intervention required for evaluation of the primary study outcome. 24 participants (n=12 in both arms) were followed-up at 24 weeks to evaluate the legacy effects of the interventions.

Baseline data are presented in Table 4.1 to include demographic information as well as imaging, metabolic tracer and cardio-metabolic parameters. These demonstrate that both study arms were well matched in respect of liver and cardio-metabolic parameters at baseline prior to commencing the study interventions with the only difference between the two groups being total and HDL cholesterol being higher in the lifestyle group.

Table 4.13: Baseline parameters for LiLi study cohort

Baseline values: mean ± SEM unless otherwise stated	**Lifestyle n=15**	**Liraglutide n=14**	**p-value**
Demographics			
Age (years)	47.7 ± 3.6	48.2 ± 3.6	0.89
Gender (% male)	50.0	53.3	0.87
Weight (kg)	104.2 ± 5.8	106.9 ± 5.6	0.59
BMI (kg/m^2)	36.4 ± 1.5	35.7 ± 1.7	0.59
Blood pressure (mmHg) systolic (S) / diastolic (D)	S: 136 ± 3.6 D: 80 ± 2.0	S: 133 ± 2.5 D: 83 ± 1.9	S: 0.43 D: 0.36
DXA body fat analysis			
Body fat (%)	45.0 ± 2.1	43.3 ± 2.5	0.48
Total fat mass (kg)	45.2 ± 3.4	45.3 ± 4.2	0.91
Total lean mass (kg)	54.7 ± 3.3	57.6 ± 3.0	0.62
Estimated visceral adipose mass (kg)	2.4 ± 0.3	2.4 ± 0.3	0.86
Magnetic resonance imaging			
MRS hepatic steatosis (%)	21.1 ± 2.4	24.3 ± 2.3	0.38
Intrahepatic iron (mg/g)	1.2 ± 0.08	1.2 ± 0.09	0.53
Iron corrected T1 (cT1)	860.6 ± 22.6	871.9 ± 28.9	0.78

(ms)			
Non-invasive markers of liver fibrosis			
Fibrosis 4 score (Fib-4)	1.1 ± 0.2	0.9 ± 0.1	0.68
NAFLD Fibrosis Score (NFS)	-1.3 ± 0.3	-1.9 ± 0.4	0.31
Plasma biochemistry			
ALT (IU/L)	61.1 ± 8.1	57.6 ± 7.6	0.57
HbA1c (mmol/mol)	37.1 ± 0.9	38.6 ± 1.2	0.26
CRP (mg/L)	5.1 ± 2.0	4.5 ± 1.0	0.8
Lipid metabolism			
Triglyceride (mmol/L)	1.5 ± 0.2	1.6 ± 0.2	0.52
Total cholesterol (mmol/L)	5.0 ± 0.3	4.1 ± 0.2	**0.014**
HDL cholesterol (mmol/L)	1.3 ± 0.09	1.0 ± 0.05	**0.0042**
non-HDL cholesterol (mmol/L)	3.7 ± 0.2	3.1 ± 0.2	0.061
Fasting de novo lipogenesis, %	12.9 ± 1.8	15.2 ± 2.5	0.65
TAG de novo lipogenesis AUC over 180min OGTT (%.min^{-1})	13.0 ± 1.9	14.4 ± 2.1	0.53
Insulin and glucose metabolism			

Fasting glucose mmol/l	4.9 ± 0.2	5.0 ± 0.2	0.35
Glucose AUC over 180 min OGTT (mmol/L.min^{-1})	7.6 ± 0.5	7.3 ± 0.4	0.77
Fasting insulin (pmol/L)	112.2 ± 18.1	135.9 ± 23.8	0.65
Insulin AUC over 180min OGTT (mmol/L.min^{-1})	897.7 ± 114.9	809.1 ± 103.6	0.47
HOMA-IR	4.2 ± 0.8	5.0 ± 0.8	0.59
Glucose oxidation (Breath $13CO_2$ AUC) over 180min OGTT (mmol/kg lean mass)	0.00024 ± 2.0x10^{-5}	0.00022 ± 1.6x10^{-5}	0.31
Physical activity			
7 day pedometer diary (steps per day)	5330 ± 686	5109 ± 732	0.80
IPAQ, time sitting (hours per day)	7.4 ± 1.2	6.6 ± 0.9	0.58
IPAQ, total weekly activity (hours)	11.8 ± 3.5	17.1 ± 5.1	0.99

4.3.2 Comparison of interventions between baseline and 12 weeks

4.3.2.1 Achievement of matched weight loss

On designing the trial to examine the weight independent effects of each intervention, it was envisaged that the interventions in both study arms would each facilitate similar weight reduction of around 5 %. In this regard, the study design was successful. The lifestyle arm lost a mean of 4.7 kg (4.4%) and the Liraglutide arm lost a mean of 5.2 kg (4.9%) with no

significant difference between arms (p=0.63). As such, this permitted direct comparison of weight-independent changes in the parameters outlined between both arms. All subjects lost weight except for one subject in the lifestyle arm.

Figure 4.18: Weight change in both arms: (a) week 0 to week 12, (b) change week 0 to week 12 and (c) number of subjects who lost weight between week 0 to week 12

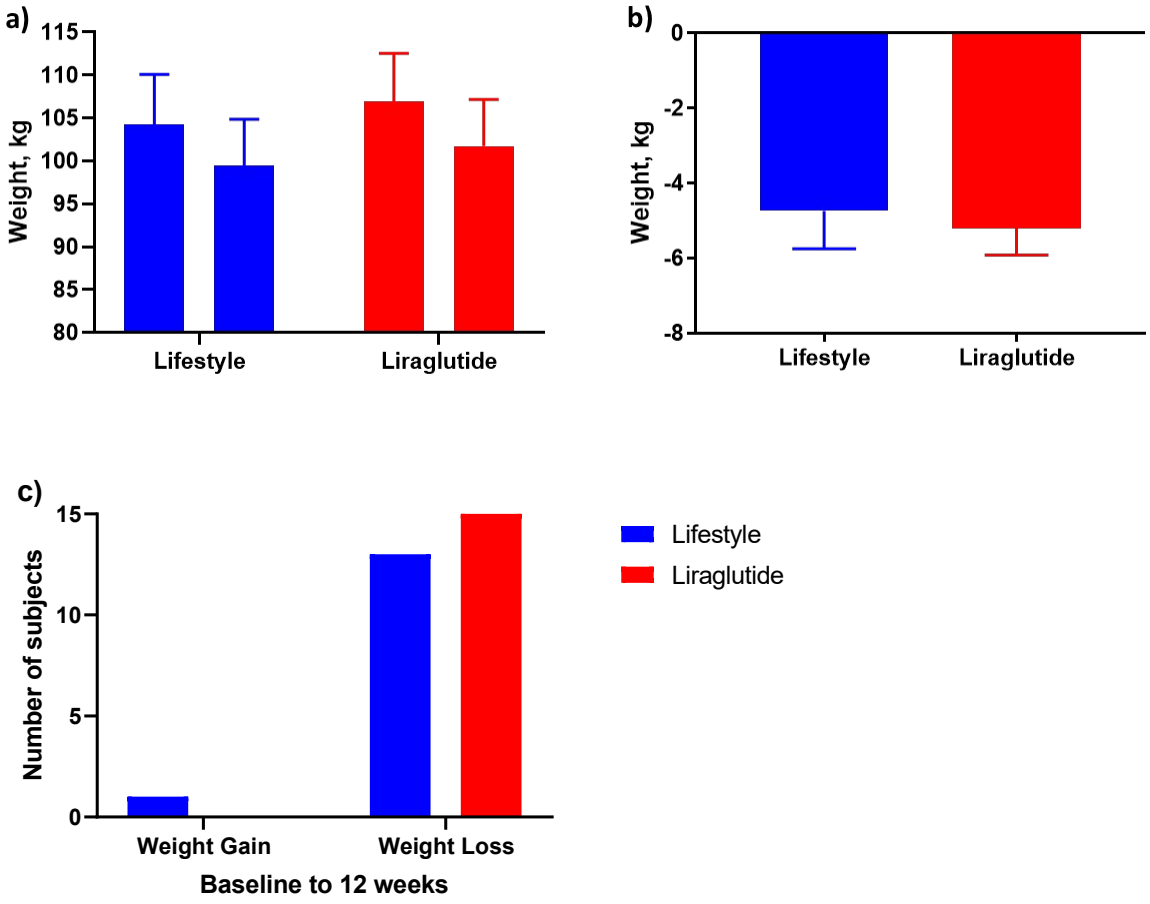


Table 4.2 outlines the changes in liver and cardio-metabolic parameters measured between baseline (week 0) and 12 weeks in both arms of the study.

Table 4.14: LiLi study: changes in parameters from baseline to 12-weeks

Change in parameters, mean ± SEM unless otherwise stated	**Lifestyle**			**Liraglutide**			**p-value**
	Baseline	**12 weeks**	**Change**	**Baseline**	**12 weeks**	**Change**	
Weight (kg)	104.2	99.5	-4.7 ± 1.0	106.9	101.7	-5.2 ± 0.7	0.63
BMI (kg/m^2)	36.4	34.8	-1.6 ± 0.3	35.7	33.9	-1.7 ± 0.2	0.48
Blood pressure (mmHg), systolic (S) / diastolic (D)	135.7 80.2	122.9 71.1	S: -12.8 ± 2.6; D: -9.1 ± 1.9	133.0 83.0	125.9 79.2	S: -7.1 ± 3.2 D: -3.8 ± 2.6	S: 0.17 D: 0.14
DXA body fat analysis							
Body fat (%)	45.0	43.3	-1.7 ± 0.4	43.3	41.9	-1.3 ± 0.3	0.39
Total fat mass (kg)	45.1	41.9	-3.2 ± 0.8	45.3	41.9	-3.4 ± 0.5	0.59
Total lean mass (kg)	54.7	54.1	-0.6 ± 0.4	57.6	56.2	-1.4 ± 0.4	0.15
Estimated visceral adipose mass (kg)	2.4	2.1	-0.3 ± 0.1	2.4	2.0	-0.3 ± 0.1	0.47
Magnetic resonance imaging							
MRS hepatic steatosis (%)	21.1	13.6	-7.5 ± 1.5	24.3	18.1	-6.2 ± 0.9	0.31
Intrahepatic iron (mg/g)	1.2	1.1	-0.1 ± 0.02	1.2	1.2	-0.0 ± 0.1	0.48
Iron corrected T1 (cT1) (ms)	860.6	813.2	-47.3 ± 16.0	871.9	827.4	-44.6 ± 12.9	0.81

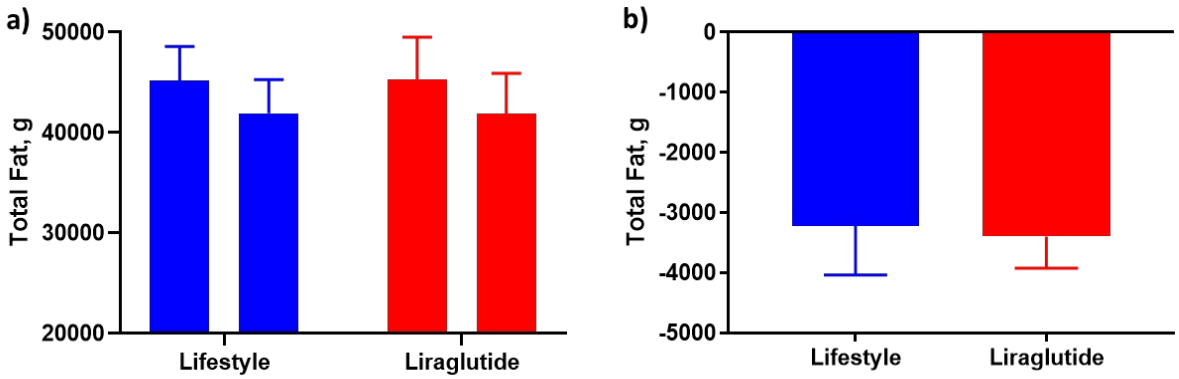
Non-invasive markers of liver fibrosis							
Fibrosis 4 score (Fib-4)	1.1	1.1	-0.0 ± 0.1	0.9	0.9	-0.1 ± 0.1	0.70
NAFLD Fibrosis Score (NFS)	-1.3	-1.2	0.1 ± 0.1	-1.9	-1.9	0.0 ± 0.1	0.59
Plasma biochemistry							
ALT (IU/L)	61.1	46.6	-14.6 ± 3.1	57.6	45.8	-15.0 ± 3.0	0.97
HbA1c (mmol/mol)	37.1	36.0	-1.1 ± 0.69	38.6	34.5	-4.1 ± 0.92	**0.014**
CRP (mg/L)	5.1	5.2	0.2 ± 0.4	4.5	4.1	-1.0 ± 0.7	0.39
Lipid metabolism							
Triglyceride (mmol/L)	1.5	1.3	-0.2 ± 0.1	1.6	1.2	-0.5 ± 0.1	0.13
Total cholesterol (mmol/L)	4.8	4.6	-0.2 ± 0.1	4.2	3.9	-0.3 ± 0.1	0.49
HDL cholesterol (mmol/L)	1.3	1.2	-0.1 ± 0.0	1.0	1.0	0.0 ± 0.0	0.069
non-HDL cholesterol (mmol/L)	3.7	3.5	-0.1 ± 0.12	3.1	2.8	-0.3 ± 0.1	0.27
Fasting de novo lipogenesis, %	12.9	10.7	-2.2 ± 1.7	15.2	9.7	-5.5 ± 2.5	0.26
De novo lipogenesis AUC over 180min OGTT (%.min^{-1})	13.0	11.5	-1.5 ± 1.9	14.4	10.2	-4.2 ± 1.8	0.18

Insulin and glucose metabolism							
Fasting glucose (mmol/L)	4.9	4.9	-0.0 ± 0.1	5.0	4.6	-0.4 ± 0.2	0.25
Glucose AUC over 180 min OGTT (mmol/L.min^{-1})	7.6	7.4	-0.2 ± 0.3	7.3	6.0	-1.3 ± 0.3	**0.023**
Fasting insulin (pmol/L)	112.2	91.3	-20.8 ± 7.3	135.9	124.6	-11.3 ± 17.1	0.084
Insulin AUC over 180min OGTT (pmol/L.min^{-1})	897.7	815.2	-82.6 ± 62.5	840.0	795.5	-44.5 ± 74.5	0.68
HOMA-IR	4.2	3.3	-0.9 ± 0.3	5.0	4.3	-0.7 ± 0.7	0.27
Glucose oxidation (Breath $13CO_2$ AUC) over 180min OGTT (mmol/kg lean mass)	0.00024	0.00023	-1.5x10^{-5} ± 8.1x10^{-6}	0.00022	0.00021	-0.7x10^{-5} ± 7.1x10^{-6}	0.53
Physical activity measures							
7 day pedometer diary (steps per day)	6160	5474	-685 ± 627	4991	5378	388 ± 316	0.27
IPAQ, time sitting (hours per day)	7.9	7.8	-0.1 ± 0.6	6.8	7.1	0.3 ± 1.1	0.62
IPAQ, total weekly activity (hours)	12.2	9.2	-2.9 ± 3.1	15.3	14.2	-1.1 ± 4.2	0.71

4.3.2.2 Anthropometry and DXA body composition

Table 4.2 outlines that weight, BMI and blood pressure all improved in both arms with no significant differences in the degree of improvement (change) between arms (p-values outlined in table). In line with total weight loss as outlined, DXA data revealed that this was primarily from a reduction in total fat mass though there was also a significant contribution from a reduction in lean mass. There were no significant differences in the degree of change of total fat %, total fat mass, total lean mass nor estimated visceral adipose mass between arms (see table for p-values).

Figure 4.19: DXA total fat: (a) week 0 to week 12, and (b) change week 0 to week 12



4.3.2.3 Physical activity

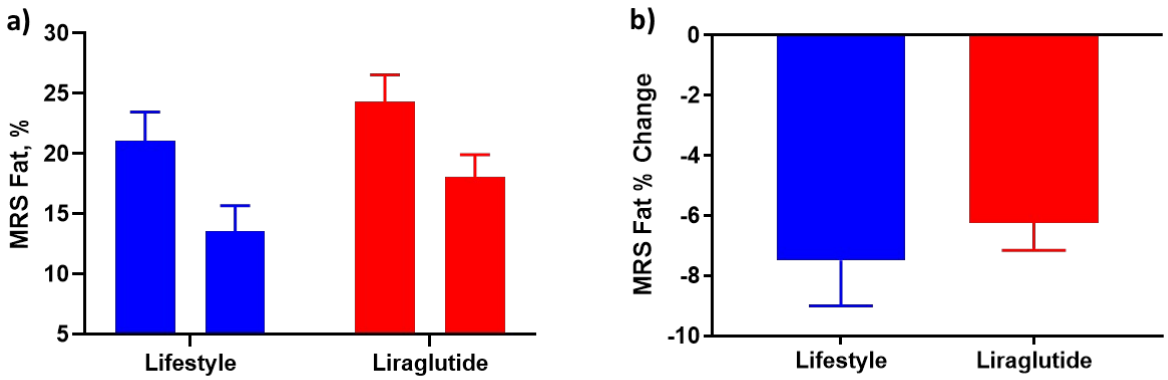
At baseline, there were no significant differences in physical activity levels as assessed by both IPAQ questionnaire and pedometer data. On analysis of change in physical activity between baseline and 12 weeks, there were no significant differences between arms (see table 4.2 for p-values).

4.3.2.4 Liver fat and liver inflammation

At baseline, both study arms had raised liver fat levels as measured by MRS (Lifestyle, 21.1 % vs Liraglutide, 24.3 %) (Table 4.1) which was well above the 5.5 % threshold for the determination of

NAFLD. Absolute liver fat reduced with weight loss (-7.5 % Lifestyle, -6.2 % Liraglutide) with no significant difference in degree of change between arms (p=0.31).

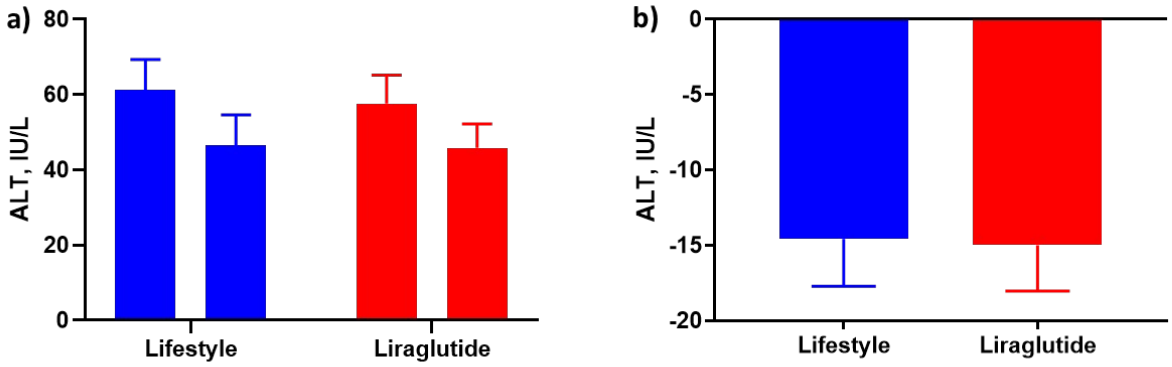
Figure 4.20: MRS fat: (a) week 0 to week 12, and (b) change week 0 to week 12



11 (79 %) of subjects in the lifestyle arm had >30 % relative liver fat reduction and 7 (50 %) of subjects in the Liraglutide arm had >30 % relative liver fat reduction between baseline and 12 weeks (Appendix 7.2, Figure 7.1). There was no significant difference in the proportion of subjects who achieved a 30 % relative reduction in liver fat in either arm (p=0.24).

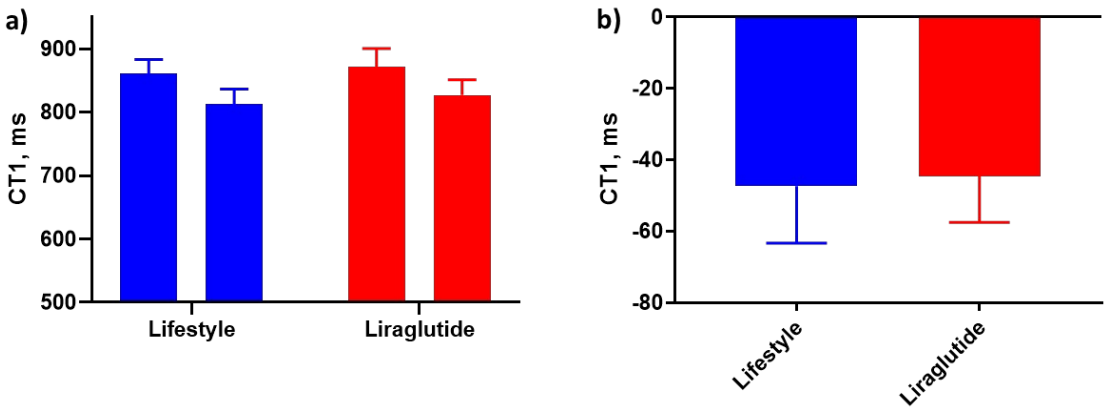
ALT as a measure of hepatic inflammation improved in both arms in conjunction with weight loss. There was no significant difference in change between arms (-14.6 IU/L and -15.0 IU/L respectively, p=0.97). The same proportion - 6 subjects (43 %) – in either arm of the study experienced a reduction of ≥17 IU/L in ALT between baseline and 12 weeks (p>0.999).

Figure 4.21: ALT: (a) week 0 to week 12, and (b) change week 0 to week 12



Liver cT1, a marker of liver inflammation and fibrosis measured using LiverMultiScan was not significantly different between arms at baseline. At baseline, both groups had a cT1 time of >800 ms which is regarded as the threshold for significant liver fibrosis. cT1 reduced with weight loss in both arms. There was no significant difference in absolute change between arms (-47.3 ms Lifestyle, -44.6 ms Liraglutide p=0.81). There was no significant difference in hepatic iron change between groups between baseline and 12 weeks (p=0.48).

Figure 4.22: cT1: (a) week 0 to week 12, and (b) cT1 change: week 0 to week 12



4.3.2.5 Glucose, insulin handling and metabolism

Fasting glucose was no different between arms at baseline (4.9 mmol/L Lifestyle versus 5.0 mmol/L Liraglutide) and was within the normal physiological range of <5.6 mmol/L in both arms. There was

no significant difference in change between groups (-0.0 mmol/L Lifestyle vs -0.4 mmol/L Liraglutide, p=0.25).

There was a significant difference in overall glycaemic control as measured by HbA1c between interventions, with a reduction of 4.1 mmol/mol in the Liraglutide arm compared with a reduction of 1.1 mmol/mol in the lifestyle arm (p=0.014).

In keeping with improvement in HbA1c in the Liraglutide arm, there was a significant difference in AUC glucose between the 2 arms over the OGTT. This showed a significantly improved glucose metabolism and disposal as measured by a greater diminished AUC in the Liraglutide arm (-0.2 mmol/L.min^{-1} Lifestyle vs -1.2 mmol/L.min^{-1} Liraglutide, p=0.02).

Glucose oxidation as measured by $^{13}CO_2$ appearance in breath over the OGTT improved with a diminished AUC in both arms between baseline and 12 weeks. As subjects in both arms lost weight between baseline and 12 weeks, yet the $^{13}CO_2$ tracer administered at all OGTTs remained fixed at 0.75 g, there was an absolute reduction in the tracer AUC measured across the OGTT. There was no significant difference in degree of improvement in glucose oxidation between arms (p=0.53).

Both groups exhibited improvement in insulin resistance as measured by HOMA-IR, though there was no significant difference in the degree of change between groups (see Table 4.2 for values and p-values). Similarly, fasting insulin levels reduced and AUC insulin diminished in both arms between baseline and 12 weeks though there were no significant differences in the degree of changes observed between arms (see Table 4.2 for values and p-values).

There was no difference in change in total body inflammation as measured by CRP between arms.

4.3.2.6 Lipid Metabolism

There was an absolute reduction in total triglycerides, total cholesterol and non-HDL cholesterol between baseline and 12 weeks in both arms though there were no significant differences in the degree of change between arms (see table 4.2 for values and p-values).

Fasting de novo lipogenesis as measured by heavy water uptake into TAG-palmitate reduced in both arms between baseline and 12 weeks (-2.2 % Lifestyle, -5.5 % Liraglutide) though there was

no significant difference in the degree of reduction (p=0.26). Similarly, DNL as measured by AUC over the OGTT showed a reduction in both arms between baseline and 12 weeks with no significant difference in the degree of reduction between arms (see Table 4.2 for values and p-values).

4.3.3 Comparison of interventions between baseline and 24 weeks

24 subjects (12 in each arm) were followed up at 24-weeks for repeat assessment to determine any legacy effects of either intervention. Tables 4.3 and 4.4 outline and compare changes in parameters between baseline, 12 and 24-weeks in these participants.

Table 4.15: LiLi study: changes in parameters from baseline to 24-weeks

Change in parameters, mean ± SEM unless otherwise stated	**Lifestyle n=12**			**Liraglutide n=12**			**p-value**
	Baseline	**12 weeks**	**24 weeks**	**Baseline**	**12 weeks**	**24 weeks**	
Weight (kg)	105.7 ± 6.6	100.4 ± 6.2	100.0 ± 5.9	103.6 ± 4.6	97.9 ± 4.4	99.3 ± 4.1	0.58
Magnetic resonance imaging							
MRS hepatic steatosis (%)	22.8 ± 2.3	13.8 ± 2.3	15.5 ± 2.7	24.7 ± 2.7	18.7 ± 2.2	21.1 ± 2.9	0.14
Iron corrected T1 (cT1) (ms)	876.4 ± 23.3	819.1 ± 27.1	821.7 ± 29.3	855.1 ± 32.6	815.2 ± 28.6	840.6 ± 26.9	0.16
Plasma biochemistry							
ALT (IU/L)	63.4 ± 9.1	47.5 ± 9.3	50.4 ±	59.5 ±	44.5 ± 7.3	46.0 ± 6.8	0.98

			11.7	10.1			
HbA1c (mmol/mol)	37.9 ± 1.1	36.3 ± 0.8	36.5 ± 1.0	39.5 ± 1.3	34.6 ± 0.7	37.1 ± 1.0	0.056
Lipid metabolism							
Fasting DNL, %	11.7 ± 2.0	9.4 ± 2.2	14.1 ± 2.6	15.5 ± 3.0	9.2 ± 2.7	14.6 ± 2.3	0.39
DNL AUC over 180min OGTT (%.min^{-1})	12.9 ± 2.3	10.1 ± 2.5	16.2 ± 3.3	14.8 ± 2.7	9.3 ± 2.6	13.3 ± 2.2	0.34
Insulin and glucose metabolism							
Fasting glucose mmol/L	4.9 ± 0.6	4.8 ± 0.4	4.7 ± 0.5	5.1 ± 0.6	4.5 ± 0.3	5.1 ± 0.5	**0.047**
Glucose AUC over 180 min OGTT (mmol/L.min^{-1})	7.6 ± 0.6	7.6 ± 0.5	7.1 ± 0.5	7.3 ± 0.5	6.0 ± 0.3	7.1 ± 0.5	**0.03**
Fasting insulin (pmol/L)	112.1 ± 21.9	90.5 ± 24.2	120.3 ± 32.1	155.5 ± 29.8	129.1 ± 17.9	139.0 ± 26.6	0.54
Glucose oxidation (Breath 13CO_2 AUC) over 180min OGTT (mmol/kg lean mass)	2.5x10^{-4} ± 2.0x10^{-5}	2.3x10^{-4} ± 1.6 x10^{-5}	2.6x10^{-4} ± 2.0x10^{-5}	2.2x10^{-4} ± 2.0x10^{-5}	2.1x10^{-4} ± 2.3x10^{-5}	2.3x10^{-4} ± 2.1x10^{-5}	0.44

Table 4.16: LiLi study: changes in parameters from baseline to 24-weeks

Change: 0 to 24 weeks. *Values: mean ± SEM unless otherwise stated*	**Lifestyle**	**Liraglutide**	**p-value**
Weight (kg)	-5.6 ± 1.9	-4.3 ± 1.3	0.59

Magnetic resonance imaging			
MRS hepatic steatosis (%)	-7.3 ± 1.8	-3.6 ± 1.8	0.20
Iron corrected T1 (cT1) (ms)	-54.7 ± 13.9	-14.5 ± 16.7	0.10
Plasma biochemistry			
ALT (IU/L)	-13.0 ± 6.3	-13.6 ± 5.7	0.93
HbA1c (mmol/mol)	-1.4 ± 1.1	-2.4 ± 1.1	0.53
Lipid metabolism			
Fasting DNL, %	2.4 ± 2.8	-0.9 ± 1.8	0.27
DNL AUC over 180min OGTT (%.min^{-1})	3.3 ± 3.1	-1.4 ± 1.7	0.31
Insulin and glucose metabolism			
Fasting glucose mmol/L	-0.2 ± 0.2	-0.0 ± 0.2	0.75
Glucose AUC over 180 min OGTT (mmol/L.min^{-1})	-0.5 ± 0.4	-0.3 ± 0.4	0.74
Fasting insulin (pmol/L)	-2.7 ± 12.9	-16.6 ± 15.0	0.43
Glucose oxidation (Breath $13CO_2$ AUC) over 180min OGTT (mmol/kg lean mass)	1.2x10^{-5} ± 8.3 x10^{-6}	1.5 x10^{-5} ± 8.3 x10^{-6}	0.85

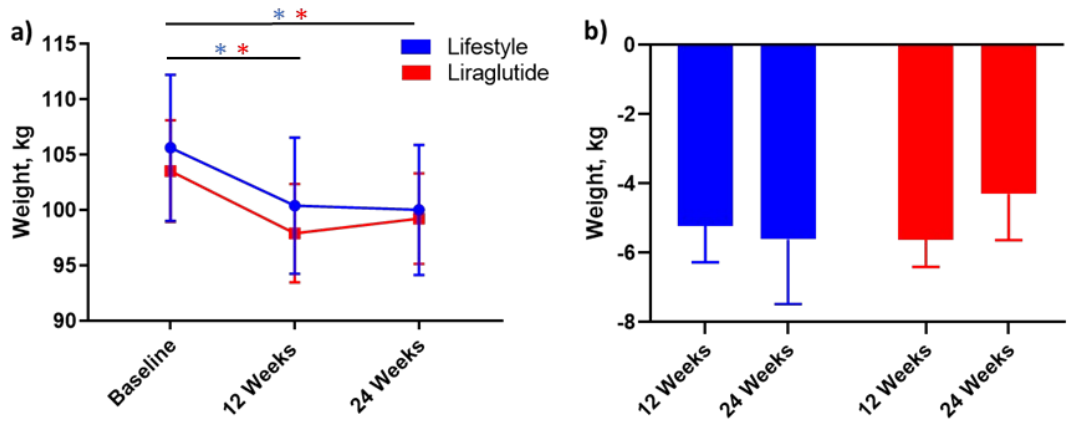
4.3.3.1 Weight

There was no significant difference in weight between arms at baseline, 12 and 24-weeks. Following initial weight loss in both arms between baseline and 12-weeks, mean weight in the Liraglutide arm increased by 1.3 ± 0.9 kg between 12 and 24-weeks whilst the Lifestyle arm had a slight reduction in

mean weight of -0.4 ± 1.0 kg at 24-weeks. 8 of 12 (Lifestyle) and 4 of 12 (Liraglutide) participants lost weight between 12 and 24-weeks. There were no significant differences in weight change between arms between baseline and 24-weeks (p=0.59) and between 12 and 24-weeks (p=0.20) respectively.

Figure 4.23: Weight: (a) baseline to 24-weeks, and (b) absolute change, baseline to 12-weeks and baseline to 24-weeks

*Significant difference (*p<0.05) between baseline and 12-weeks and baseline and 24-weeks, though no significant difference between 12 and 24-weeks in each arm separately. No significant difference comparing arms at each timepoint (a) nor when comparing changes between arms (b).*



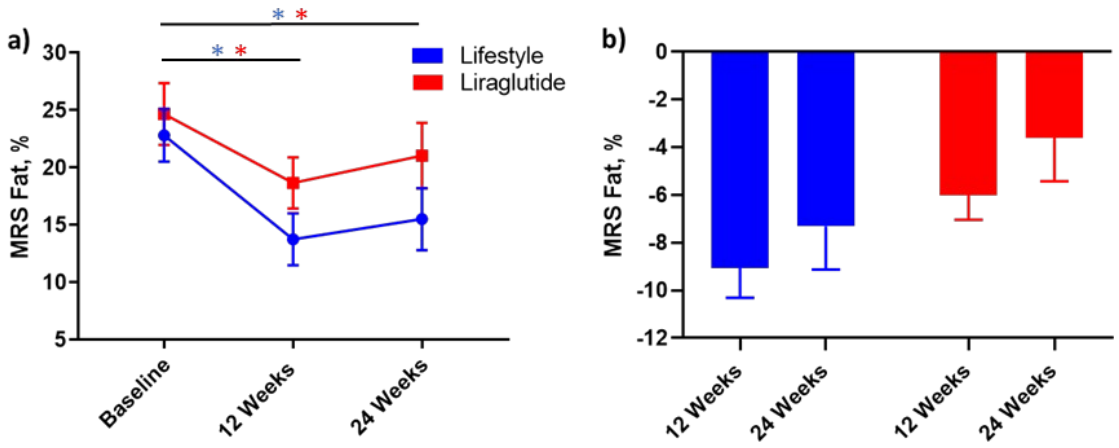
4.3.3.2 Liver fat and liver inflammation

Liver fat change as measured by MRS between baseline, 12 and 24-weeks are outlined in Tables 4.3 and 4.4 and show that there were similar magnitudes of changes with no significant differences between arms at all three time points.

There was a reduction in ALT with weight loss between baseline and 12 weeks and ALT rise between 12 and 24-weeks in line with weight gain in the Liraglutide arm and similar rise in ALT in the Lifestyle arm. There was no significant difference in the magnitude of ALT change between baseline and 24 weeks (see Table 4.4 for values and p-values).

Figure 4.24: MRS liver fat: (a) baseline to 24-weeks, and (b) absolute change baseline to 12 and 24-weeks

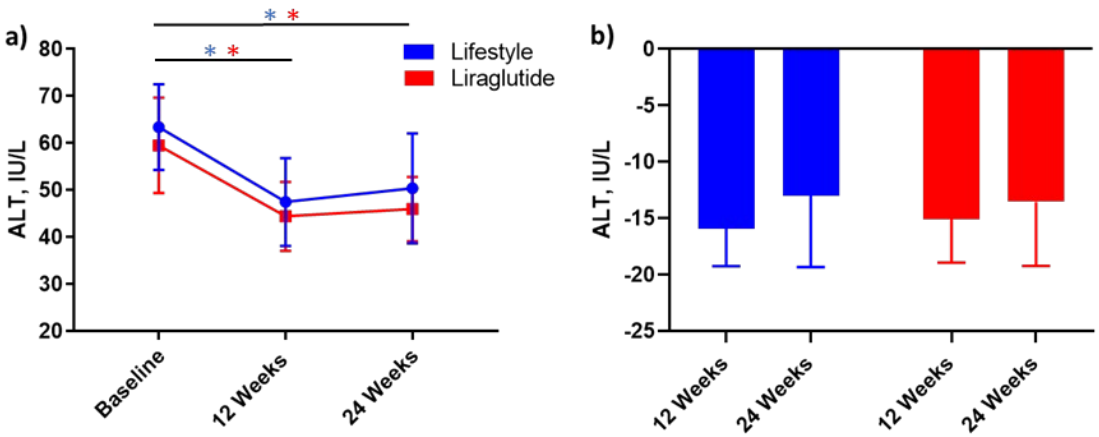
*Significant difference (*p<0.05) between baseline and 12-weeks and baseline and 24-weeks, though no significant difference between 12 and 24-weeks in each arm separately. No significant difference comparing arms at each timepoint (a) nor when comparing changes between arms (b).*



Liver inflammation as measured by plasma ALT followed the same pattern in both arms falling by 12 weeks and rising at 24 weeks. There were no significant differences between arms from baseline to 12 and 24 weeks and the degree of changes between baseline and 24 weeks was no different between arms (p=0.93).

Figure 4.25: ALT: (a) baseline to 24-weeks, and (b) absolute change baseline to 12 and 24-weeks

*Significant difference (*p<0.05) between baseline and 12-weeks and baseline and 24-weeks, though no significant difference between 12 and 24-weeks in each arm separately. No significant difference comparing arms at each timepoint (a) nor when comparing changes between arms (b).*

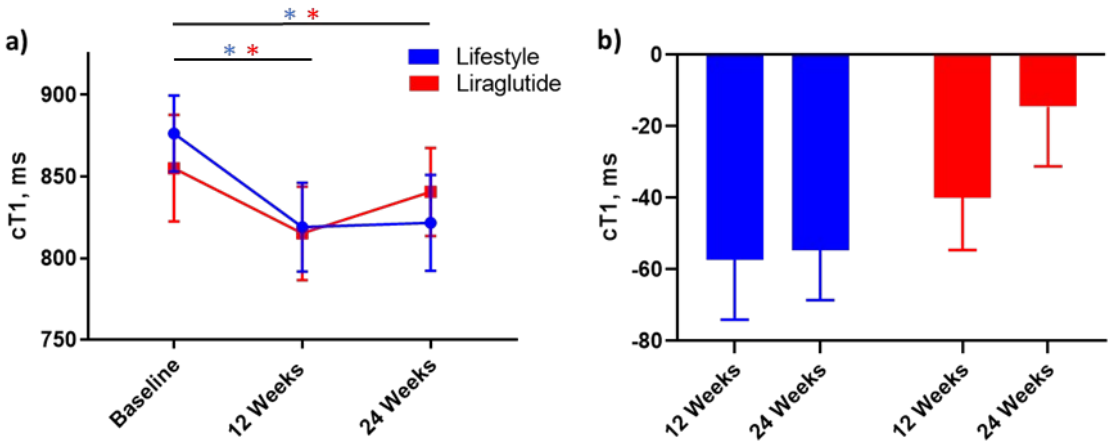


cT1 measure of liver fibrosis and inflammation showed similar changes with a reduction between baseline and 12-weeks in both arms in concordance with weight loss. cT1 rose thereafter with the weight regain observed in the Liraglutide arm. cT1 showed a slight but non-significant mean rise of

2.6 ms in the Lifestyle arm which was consistent with the 0.4 kg mean weight loss observed in this arm between 12 and 24-weeks. Comparing changes to cT1 between baseline and 24-weeks, there was a trend to greater reduction in the Lifestyle arms (-54.7 ms Lifestyle vs -14.5 ms Liraglutide , p=0.10).

Figure 4.26: cT1: (a) baseline to 24-weeks, and (b) absolute change baseline to 12 and 24-weeks

*Significant difference (*p<0.05) between baseline and 12-weeks and baseline and 24-weeks, though no significant difference between 12 and 24-weeks in each arm separately. No significant difference comparing arms at each timepoint (a) nor when comparing changes between arms (b).*



4.3.3.3 Glucose and insulin handling and metabolism

Fasting glucose was significantly different between baseline, 12 and 24-weeks (p=0.047) driven by a reduction in the Liraglutide arm that occurred between baseline and 12-weeks. By 24-weeks, on cessation of the drug, fasting glucose had returned to baseline levels in the Lirgalutide arm. There was no difference between arms when comparing the change in fasting glucose between baseline and 24 weeks (p=0.75).

In keeping with this improvement in fasting glucose in the Liraglutide arm, there was a significant difference in AUC glucose over the OGTT between the 2 arms when comparing baseline to 12 and 24-weeks (p=0.03). This was driven by improvement in glucose metabolism and disposal as measured by a greater diminished AUC in the Liraglutide arm at 12-weeks.

Figure 4.27: Fasting glucose: (a) baseline to 24-weeks, and (b) absolute change baseline to 12 and 24-weeks

*Significant difference (*p<0.05) between baseline and 12-weeks and between 12 and 24-weeks in the Liraglutide arm. No significant difference comparing arms at each timepoint (a) nor when comparing changes between arms (b).*

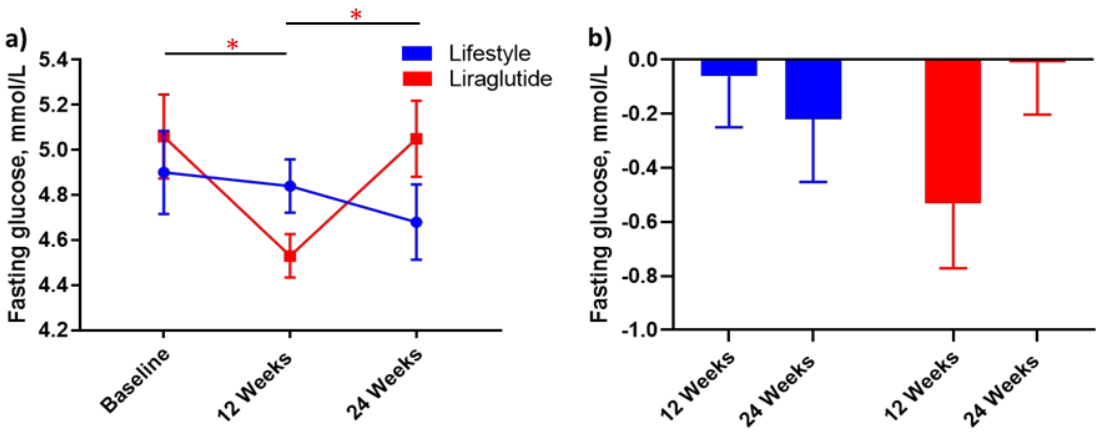


Figure 4.28: HbA1c: (a) baseline to 24-weeks, and (b) absolute change baseline to 12 and 24-weeks

*Significant difference (*p<0.05) between baseline and 12-weeks, baseline and 24-weeks and 12 and 24-weeks in the Liraglutide arm. No significant difference comparing arms at each timepoint (a) nor when comparing changes between arms (b).*

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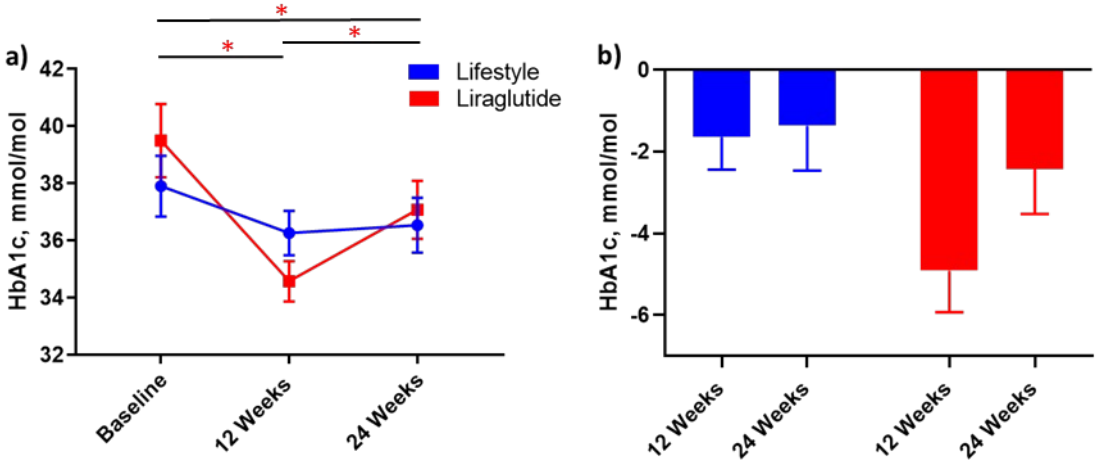


Similarly, there was a trend to a greater improvement in glycaemic control as measured by HbA1c in the Liraglutide arm (p=0.056) which was again driven by improvements seen whilst on the drug between baseline and 12-weeks. Comparing changes between baseline and 24-weeks, there was no significant difference between arms (p=0.53).

There was no significant difference between arms in glucose oxidation as measured by $^{13}CO_2$ appearance in breath between baseline, 12 and 24 weeks and no difference in the degree of change when comparing baseline to 24 weeks (see Tables 4.3 and 4.4 for values).

4.3.3.3 Lipid Metabolism

There was no significant difference between arms in fasting DNL and DNL AUC over the OGTT at baseline, 12 weeks and 24 weeks, as measured by uptake of heavy water into TAG-palmitate. Additionally, there was no significant difference in the magnitude of change of fasting DNL and DNL AUC over the OGTT when comparing baseline to 24 weeks (see Tables 4.3 and 4.4 for values).

4.4 Discussion

4.4.1 Comparison of health improvements between baseline and 12 weeks

4.4.1.1 Improvements in cardio-metabolic and liver health

This study was designed to compare the effects of weight loss induced via dietary and lifestyle changes compared to weight loss induced by the GLP-1 agonist Lirgalutide in non-diabetic subjects with NAFLD. Both arms achieved equivalent weight loss by 12-weeks (4.4 % Lifestyle vs 4.9 % Liraglutide) which was an amount known to confer improvement in cardio-metabolic and general health and which permitted a direct comparison of any weight independent improvements in health.

In accordance with the magnitude of weight loss observed in both arms of the study, there were absolute improvements in cardio-metabolic health parameters including blood pressure, glycaemic control, lipid profiles including triglycerides and total cholesterol in both arms. Absolute

improvements were also observed when assessing changes to liver health parameters with both groups recording absolute improvements in liver fat content as measured by MRS and in liver inflammation as measured by ALT and cT1.

Weight loss was primarily achieved via a reduction in total fat mass as measured by DXA scanning though there was also a reduction in lean mass in both groups. Weight loss in both arms was achieved through a reduction in total calorie intake, with physical activity measures (pedometer data and exercise questionnaires) showing no major increase in physical activity in either arm.

4.4.1.2 Comparison of health improvements between baseline and 12 weeks

4.4.1.2.1 Liver fat and inflammation

This study is in line with previous literature which shows that with an approximate 5 % weight loss in patients with NAFLD treated with Liraglutide, there is a corresponding reduction in liver fat content and a corresponding improvement in ALT (170,340–342). Furthermore, the reduction in liver fat content of 6.2 % seen in the Liraglutide arm is in the order of magnitude reported in previous studies in patients with NAFLD treated with Liragluitde (340,343). Similarly, a meta-analysis of lifestyle interventions for NAFLD which analysed studies with weight loss of between 4-14 % showed a corresponding reduction in liver fat of 4-10 % (344). These figures are again in line with my study which shows a 7.5 % reduction in the lifestyle arm.

Studies to date have found that improvements in liver fat and inflammation appear to primarily be driven by weight reduction (342,344). Indeed, in my study, using a mixed effects model on all paired data irrespective of intervention (See Chapter 7, Appendix 2.) to investigate the primary drivers of improvement in MRI liver fat in all subjects, weight was the only factor identified which governed the reduction in liver fat observed. Furthermore, in a similar analysis of improvement in cT1 as a marker or liver inflammation and fibrosis, weight was found to be the most important factor governing improvement in cT1.

This study demonstrated significant improvements in liver fat as measured by MRS, liver inflammation as measured by ALT and inflammation and fibrosis as measured by liver cT1 between baseline and follow-up. It did not however show a change in Fib-4 or NFS in either arm. Previous

studies have however shown that both Fib-4 and NFS can correlate with changes in liver fibrosis over time in patients with NAFLD (29,120) and patients with hepatitis C (345), though these studies presented data from longer follow up periods compared to my study.

The reasons for the discrepancy in no change in Fib-4 and NFS compared to cT1 may be due to the relatively small study numbers due to this being an academic study. The study subjects could also more generally have had earlier stage fibrosis and as Fib-4 and NFS perform best in excluding advanced or significant fibrosis, perhaps they reflected this in their performance. Additionally, approximately half of subjects (43 %) in each study arm experienced a 17 IU/L reduction in ALT which is thought to predict histological liver response as highlighted in data from the FLINT study investigating obeticholic acid in the treatment of NASH (278). Thus perhaps the Fib-4 and NFS scores reflect a finding that my study interventions may not have had a direct impact on liver fibrosis in all subjects by 12 weeks. Liver fat and liver inflammation are more likely to have improved by 12 weeks and as cT1 is described as a measure of both liver inflammation and fibrosis, it is possible that perhaps the significant improvement observed in this parameter at 12 weeks reflects changes to inflammation more so than to fibrosis. On a similar note, an approximate 30 % reduction in liver fat, as was achieved by 79 % of subjects in the lifestyle arm and 50 % of subjects in the Liraglutide arm has been shown to predict high rates of NASH (inflammation) resolution (346,347).

4.4.1.2.2 Improvements in cardio-metabolic health and in lipid, insulin and glucose metabolism

Reinforcing the finding that weight loss is the most important determinant of health improvements in subjects with NAFLD in this study, direct comparison of the magnitude of improvements observed in cardio-metabolic health between the 2 arms found that there were few significant differences in improvements.

With regards to lipid metabolism, there was no difference between lifestyle and Liraglutide arms in fasting lipid profiles nor in de novo lipogenesis – either fasted or dynamically across an OGTT – as measured as the percentage of uptake of deuterated water into triacylglycerol palmitate. At baseline, de novo lipogenesis was similar in the 2 groups and being of a magnitude of 13-15 %, was similar to that previously reported in the literature of around 15-25 % in patients with NAFLD (73,74). With weight loss, DNL reduced in both groups to around 10 %, which is again consistent with literature

that shows that patients with NAFLD with lower levels of liver fat had as a percentage of triacylglycerol palmitate, de novo lipogenesis rates of around 10 % and that in general, patients with NAFLD have higher DNL rates than those without NAFLD (73,348).

Additionally, there was no significant difference in the magnitude of changes in insulin handling and metabolism between arms, measured in either the fasted state, or when measured dynamically over an extended 180 min OGTT. There was however a significant increase in glucose disposal in the Liraglutide arm at 12 weeks measured by a reduction in AUC glucose over the OGTT. This is consistent with literature which shows that the blood glucose lowering actions of GLP-1 are glucose-dependent and that GLP-1 agonists reduce glucose levels by stimulating insulin secretion from pancreatic β-cells by activating the glucagon-like peptide 1 receptor only when glucose levels are elevated above fasting levels such as in the post-prandial state, which in this study was mimicked by the OGTT (349,350). There was however no corresponding rise seen in either fasting insulin nor across the OGTT in this study in either arm. Nevertheless, participants in this study, although all non-diabetic at baseline, demonstrated severe insulin resistance as measured by HOMA-IR (351) at levels which were considerable higher than levels previously suggested (HOMA-IR 2.0-2.5) as showing diagnostic value in distinguishing NAFLD from health controls (352). By 12 weeks, with weight loss, absolute insulin resistance had improved (though both arms remained severely insulin resistant), findings which were consistent with a reduction in fasting insulin levels and in insulin levels measured across the OGTT. In contrast to glucose metabolism, the magnitude of changes to insulin handling and metabolism were not significantly different between arms.

Furthermore, as expected and in line with previous findings in subjects with fatty liver disease and or diabetes (353,354), there was an increase in glucose metabolism and greater efficiency in glucose oxidation with weight loss at 12 weeks compared to baseline in both groups, as measured by the appearance of $^{13}CO_2$ in breath. Interestingly however, and in contrast with glucose disposal over the OGTT, there was no significant difference in the magnitude of this change with both arms demonstrating equally improved glucose oxidation. This suggests that the increased glucose disposal seen in the Liraglutide arm across the OGTT was pehaps not as a result of increased glucose oxidation.

4.4.2 Assessment of legacy effects of interventions at 24 weeks

The data arising from this study suggest that there are no obvious direct liver or cardio-metabolic legacy effects beyond inducing weight loss arising from either Liraglutide therapy or lifestyle intervention when compared with each other

On stopping each intervention, there was a 23 % regain of initial weight lost in the Liraglutide arm, and maintenance of weight loss in the lifestyle arm (there was minor though no significant weight loss of 0.4 kg in the lifestyle arm). As there were no significant changes to physical activity levels between groups, the explanation for this difference is likely to be the conscious efforts and behaviour modifications made to dietary intake made by those in the lifestyle arm. These dietary behavioural changes are likely to have persisted despite the formal cessation of the lifestyle intervention resulting in the weight maintenance observed whilst those on Liraglutide appear to have quickly lost the appetite suppression induced by Liraglutide on cessation of therapy.

Health parameters including liver fat and liver inflammation largely mirrored weight findings with no significant changes in values from 12 to 24 weeks, except for a return to baseline in values of glucose disposal and glycaemic control on cessation of Liraglutide. This data on glucose handling, especially when considered in the context of the generalised study findings, provides credence to the hypothesis firstly that weight loss irrespective of how it is achieved is the key to improvement in fatty liver disease and secondly that the benefit of medication only persists whilst on therapy and ceases on termination. In comparison, this study suggests that dietary lifestyle intervention is able to maintain weight loss on stopping of formal lifestyle intervention.

It is important to note however that the follow-up duration of 3 months after cessation of intervention in this study is short and ideally a longer term follow-up data would be beneficial as it is well known that after initial success at weight loss, weight loss maintenance is challenging. Previous studies show that individuals who achieved weight loss via diet and exercise had weight regain of around 55 % of initial weight lost by 18 months (355). Furthermore, data emerging from long term follow-up of patients who have undergone bariatric surgery, which is currently the most effective weight loss intervention, suggest that individuals may regain 15-25 % of initial weight lost over time (356,357). One previous study looking at weight loss in those who completed 6 months of GLP-1 therapy showed that subjects regained around 60% of initial weight lost at 6 months after stopping therapy,

whilst in the same study, those who lost weight via dietary and lifestyle measures maintained weight loss at 6 months (341). These result are similar to my study which shows that the Lifestyle arm had maintenance of weight loss whereas the Liraglutide arm had 23 % weight regain at 3 months. There are however, as yet, no studies on outcomes from both longer-term GLP-1 use in this context nor long-term follow-up data after GLP-1 therapy cessation in NAFLD.

4.4.3 Future role of GLP-1 therapy in treating NAFLD

This study clearly demonstrates that GLP-1 therapy and lifestyle intervention had equivalent efficacy with respect to NAFLD improvement whilst on intervention and as such showed that GLP-1 therapy has clear value in treating NAFLD in certain circumstances. Furthermore, the health improvements reported in both arms were attained without any significant change in physical activity levels. This is important in those who are unable to achieve or sustain weight loss with diet and or exercise interventions. In this regard, this study adds to the body of evidence that suggests GLP-1 agonists may be suitable for licencing from health authorities for use in patients with NAFLD. Once this has been achieved, it will be for clinicians in conjunction with health planners to decided how GLP-1 therapy should be used in patients with NAFLD, for example via a stepwise approach after lifestyle measures have been tried or in patients with NAFLD who have evidence of cardiovascular complication as GLP-1 agonists are proven to have cardio-protective benefits (322).

Cost of therapy will also be an important factor in these decisions. One study estimated that around 25 % of patients cited cost factors as the reason for stopping therapy which is relevant to some non-UK health sytems (358). In the UK, it should be noted that Liraglutide at a higher dose of 3 mg (Saxenda) as opposed to the 1.8 mg dose (Victoza) used in this study has an EU licence for weight loss irrespective of diabetes, but is as yet not in routine clinical use within the NHS due to it not having NICE approval. Nevertheless, Liraglutide is likely to be cost-effective when considering overall health benefits and reductions in medical complications in patients with cardio-metabolic disease as suggested by the metabolic hepatology clinic analysis presented in Chapter 2, though further evidence is required to support this (359).

With regards to patient acceptability, Liraglutide was generally well tolerated in my study which is in line with previous data. 2 subjects withdrew due to side effects after commencing the study drug (1 experiencing gastrointestinal upset, 1 experiencing rash). Others also complained of adverse

gastrointestinal effects though were able to continue taking the therapy. Gastrointestinal adverse effects of GLP-1 therapy are well known and reported particularly at initiation, though do improve with time in those able to persevere with therapy. Other limitations of GLP-1 therapy include eventual plateauing of weight loss, and the need for daily injections due to its subcutaneous form of administration. Nevertheless, an oral GLP-1 preparation - Semaglutide – is on the horizon and showing good promise in clinical trials in regards to both efficacy and safety (360).

4.4.4 Limitations of this study

As an academic project, this was a relatively small study with small patient numbers, even though the weight loss and reduction in liver fat content planned were met at the time of analysis. Nevertheless, results will need to be interpreted with this in mind. It is also important to note that although established liver and cardio-metabolic parameters were assessed, the outcome measures did not include liver biopsy which continues to be the gold standard diagnostic measure of NASH and liver fibrosis. Furthermore, study duration which included 3 months of intervention allowed for meaningful weight loss of around 5 % and thus for in depth analysis of health improvements. This duration of therapy however was still relatively short and the rate of weight loss observed would not have plateaued at 3 months. Similarly, assessment of legacy effects at 3 months after therapy cessation may not have allowed sufficient time for health benefits to accrue and persist.

There are also further analyses arising from this study that are planned including analysis of adipose tissue samples to assess for ‘adipose tissue specific’ effects such as adipose inflammation and fat metabolism and a more detailed assessment of ‘whole body inflammation’, including plasma cytokine analysis, as both obesity and GLP-1 therapy are known to modulate inflammation. As such, work is ongoing in this regard.

4.5 Conclusion

The clinical study presented in this chapter clearly demonstrates that weight loss is key to improvement in NAFLD.As such, weight loss should be strongly promoted by clinicians to improve

the health outcomes for patients with NAFLD irrespective of how it is is achieved as this study showed that are minimal weight independent differences in health improvements observed between those randomised to Liraglutide or those randomised to lifestyle. In this regard, this study supports the view that both lifestyle interventions and GLP-1 agonists should be amongst the tools available to clinicians. Nevertheless, a stepwise approach will be of benefit, starting with trying to acheive weight loss via diet and lifestyle changes as this may help influence long term behaviour and allow maintenance of weight loss at least in the short term as demonstrated in this study.. This information will be useful for health planners when formulating health policy and making licensing decisions for new therapies with which to treat NAFLD.

Chapter 5 Final conclusions and future perspectives

5.1 Final conclusions

5.1.1 The need to tackle the global NAFLD epidemic

NAFLD is an important global health epidemic which needs to be urgently tackled to reduce patient morbidity and mortality as well as to reduce the rising health-economic burden it brings to society (4). NAFLD is still an underappreciated entity by healthcare professionals (106) though with time and increased awareness this is slowly changing. To help healthcare providers tackle this epidemic, new approaches to diagnosis and management are required, particularly as NAFLD is predicted to be the leading cause of morbidity and mortality in liver disease over the coming years in addition to exerting its principal complications through cardio-metabolic disease (3). Chapter 1 explained the health complications and burdens in detail to explain why NAFLD poses such significant challenges to health systems and what has been done to date both in regards to diagnosis, risk stratification and management and also explained why the current the tools available to the clinician in this regard are imperfect (1,114,115).

5.1.2 The need for better designed health services for the management of NAFLD

In recognition of the fact that current best practice models for NAFLD are in their infancy and remain to be fully defined, Chapter 2 described and evaluated the outcomes from one interventional approach delivered through a dedicated multidisciplinary metabolic hepatology clinic aimed at tackling the NAFLD within Oxfordshire, UK. This chapter described how this service is run jointly by hepatologists and diabetologists working together alongside allied health professionals including dietitians and lifestyle experts to provide a holistic model of care for patients. This model incorporates key features of guidelines published to date (104,147,148). Importantly, the evaluation of the approach undertaken in Chapter 2, and in particular of clinical outcomes since inception of the service in 2014, demonstrated that this approach resulted in meaningful improvements to surrogate markers of both liver and cardio-metabolic health. Similar improvements have previously been described (264) which adds weight to the credence of this approach, though, further to this, my

study yielded evidence for the first time that liver stiffness measured using hepatic elastography improved in a real life clinical setting in patients with NAFLD.

It should be noted however, that the improvements demonstrated in my study were shown in a cohort followed for a relatively short median duration of 13 months, and that as this was an analysis of clinical practice data, there was no dedicated 'usual care' or control group for comparison. Nevertheless, if the clinical improvement shown are sustained longer term, they will serve to reduce future NAFLD related complications and reduce the future burden of disease, which was evidence by the significant reduction in 10 year cardio-vascular risk seen as measured using the QRISK-3 score. Furthermore, the economic modelling undertaken in this cohort of patients with diabetes was suggestive of cost effectiveness longer term which will help to reduce future economic burden on health systems whilst improving health outcomes. Consequently, these results are relevant to both physicians as direct care providers to patients to help inform their clinical practice, and are useful for health policy planners in providing evidence to support the development of future NAFLD healthcare services.

Future work is planned to follow this cohort from the metabolic hepatology clinic for a longer duration of follow-up and I hope with colleagues to provide data from future analyses in due course.

5.1.3 Appropriate risk stratification of patients with NAFLD is of high importance

Due to the high prevalence and burden of disease – NAFLD affects up to 30 % of the unselected population (361) – when designing future health services for NAFLD, it will be impractical for all patients to be reviewed and managed by specialists in secondary care. As such, management of most patients with NAFLD will need to be performed within primary care settings. In order for this to be effective and to be able to triage patients to the most appropriate care setting (primary, secondary or tertiary care), there is an important need for the availability of robust and accurate screening, diagnostic and risk stratification tools. Chapter 1 described how liver biopsy, the current gold standard risk stratification tool is both invasive and impractical in this regard and reviewed current non-invasive tools which are being used in this regard, which are again imperfect (1,114,115).

The study presented in Chapter 3 is an exciting, wholly novel approach to non-invasive risk stratification using the urinary steroid metabolome. The study firstly showed that analysis of the urinary steroid metabolome using machine learning techniques had excellent potential in distinguishing healthy controls from those with NAFLD. As this analysis was undertaken in spot urine samples which can be easily and routinely collected in primary care, there is good potential for use of this technique as a future routine screening tool for NAFLD, for example as part of the UK NHS routine health check (362), at the annual health check all patients with diabetes should receive (363), or for others at high risk such as those with obesity.

After initial diagnosis, the ability to accurately distinguishing early fibrosis from advanced fibrosis is the key risk stratification question which determines the level of management and monitoring required and whether management should occur in primary or secondary care. There is currently no universally accepted non-invasive risk stratification technique and referral to secondary care for liver biopsy, which remains the gold standard, is often required. In this regard, Chapter 3 demonstrated that accurate non-invasive risk stratification of early from advanced fibrosis using the urinary steroid metabolome is possible and again holds excellent promise. Further to this, the urinary steroid metabolome was also able to accurately distinguish cirrhotic from non-cirrhotic NAFLD. As liver cirrhosis has a high chance of progression to hepatocellular cancer, patients with cirrhosis are managed differently from those without cirrhosis, for example with gastroscopy and liver ultrasound monitoring. This study shows that cirrhosis surveillance practices could potentially be enhanced through use of the urinary steroid metabolome. Previous studies in the context of adrenal cancer demonstrated the feasibility of the urinary steroid metabolome to distinguish malignant from benign lesions (316), and, as such, applying this technique in the context of liver disease in future studies may yield further potential uses.

Nevertheless, despite the very promising results, it must be noted that the urinary steroid tool presented requires validation in a separate larger cohort and may not be the perfect test. If successfully validated and depending on future performance of the test, it may be that future liver services incorporate this test with other existing non-invasive tools such as the Fib-4 score (117) or the NAFLD Fibrosis Score (118) to help risk stratify patients. Additionally, this ‘combination approach’ may be complemented by other techniques including multiparametric MR Liver*MultiScan* described in Chapter 1 and used in the clinical trial outlined Chapter 4. Excitingly, in Chapter 4, I

demonstrated for the first time that in subjects with GLP-1 or lifestyle induced weight loss, that Liver*MultiScan* and in particular the cT1 measure of liver inflammation and fibrosis, is able to track liver disease change, in this case regression, as shown by the reduction in cT1 at follow-up, which highlighted its value as a potential monitoring tool. This finding in keeping with a recent study that showed cT1 reduced with a reduction in liver fat (229).

It should also be noted that the Fib-4 and NFS scores already incorporate a combination of clinical parameters such as age along with serological parameters such as ALT, and that recent data has shown value in combining cT1 with serological data to improve assessment of liver inflammation (364). Similarly, in the urinary steroids study, the computational analysis employed an approach which combined urinary steroid data with age and BMI data. In future studies, in addition to validating findings thus far in a larger cohort, I plan to combine serological and urinary steroid data to assess for improvement in diagnostic performance. Indeed, this type of 'combination biomarker approach' may be the most pragmatic option in future to reduce reliance on liver biopsy.

Finally, in regards to future research using the urinary steroid metabolome, work is already under way to assess performance of the urinary steroid metabolome in the diagnosis of hepatocellular carcinoma and I will be part of the team to report on these results in due course.

5.1.4 The need for efficacious interventions and treatment options for NAFLD

In addition to accurate risk stratification, there is a corresponding need for better targeted interventions and therapeutic options for NAFLD which are efficacious and cost-effective, and able to delivered across primary and secondary care. Whilst there are currently no specifically medical therapies for NAFLD, a number of therapies, including GLP-1 agonists are on the horizon and a number of others are under development as outlined in Chapter 1. Chapter 4 described my clinical trial investigating the GLP-1 agonist Liraglutide as one such therapeutic option and building on previous work investigating this class of drug (170,171,340,341) clearly demonstrated that these agents are able to play an important role in treating NAFLD. This study adds to the evidence base in this field by clearly demonstrating that there are minimal weight independent benefits of GLP-1 agonists compared to lifestyle induced weight loss. There were also suggestions in the study that

those who lost weight via lifestyle changes were able to maintain this weight loss but this did not reach significance perhaps due to a lack of statistical power. Accordingly, the study's conclusion is that weight loss is the key to improvement in NAFLD irrespective of how it is achieved. Nevertheless, clinicians as a first line intervention for NAFLD should recommend lifestyle changes and consider referral to weight management programmes based around lifestyle changes if possible.

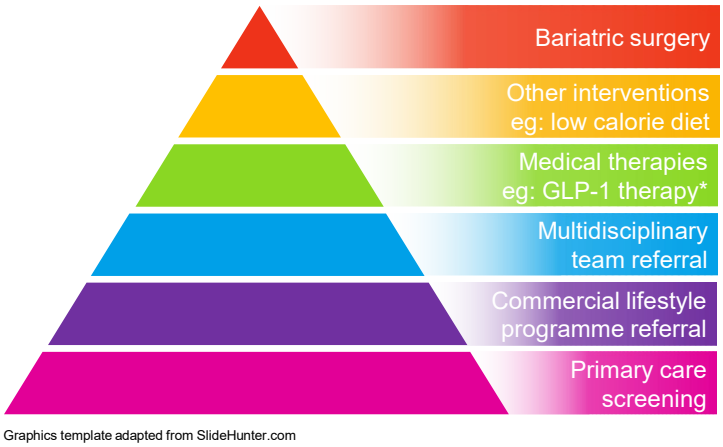
Whilst the non-superiority of Liraglutide as a therapeutic agent may at first glance perhaps disappoint, it is important to note that weight loss, however it is achieved, is difficult to maintain longer term. Consequently, clinicians will need a variety of options at their disposal with which to treat patients with NAFLD. Current clinically proven weight loss options are those which are firstly lifestyle driven, either via referral to health specialists such as dietitians or those achieved via commercial weight management programmes (151,365,366), secondly medication driven, or thirdly those achieved via weight-loss surgery. It is also worth noting that the magnitude of weight loss is in approximately the same order with newer GLP-1 agents such as Semaglutide showing that weight loss of up to 14 % is possible at one year (367). Nevertheless, weight-loss surgery remains the gold standard and most efficacious option and depending on the nature of the procedure performed, may achieve weight loss of between 20-30 %, which importantly is likely to be sustained at long term follow-up (368). When discussing weight-loss surgery, it should also be noted that low calorie diets are used in the lead up to surgery and that these diets have recently been shown to be effective in treating diabetes and NAFLD (152–154).

5.2 Future perspectives

In a future consultation for a patient with NAFLD either in primary or secondary care, after accurate risk stratification has been performed and in the knowledge that not all treatments are suitable for all patients, clinicians will wish to arrive at an individualised management plan in conjunction with patients and base treatment decisions on the particular circumstances of the individual. For example, in the knowledge that GLP-1 therapies improve diabetes and confer cardiovascular benefits to patients, a clinician may suggest this therapy early on to a patient with NAFLD and co-existing heart disease or diabetes. Alternatively, in a younger patient with NAFLD and no co-

morbidities who has been risk stratified in primary care to have early stage fibrosis, a GP may instead choose to refer a patient to a commercial weight loss programme in the first instance before progressing on to medical therapy if required. Figure 5.1 attempts to visualise one possible step-wise, layered approach to the treatment of NAFLD in future with the lower stages of the pyramid based in primary care and the higher levels perhaps based in secondary care based on risk stratification and or treatment response.

Figure 5.29: A potential weight loss management structure for NAFLD



It should also be noted that Figure 5.1 primarily refers to weight loss modalities and does not currently include any other treatment options, for example anti-fibrotic therapies. Some newer experimental agents are under development and are briefly mentioned in Chapter 1 and it would be hoped that treatment pathways of the future will contain these types of options as when they become available.

Accordingly, opportunities to make a difference and tackle the challenges in the NAFLD sphere remain plentiful. NAFLD is thus an exciting and interesting field in which to currently be involved and I personally hope I am able to continue to play a role to meet this challenge in my future academic and clinical practice as I progress further in my career.

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Chapter 7 Appendices

7.1 Appendix to chapter ‘A multi-disciplinary approach to the management of NAFLD is associated with improvement in markers of liver and cardio-metabolic health’

Incidental Finding of Abnormal Liver Function Tests

Note on Referral Guidelines: These revised guidelines are presented as a tool to aid appropriate referral and management of common liver-related problems. The revisions are in response to increasing prevalence of non-alcoholic fatty liver disease (NAFLD), incidental finding of hepatic steatosis on US scanning and our awareness that standard LFTs do not indicate severity of NAFLD, hence we are targeting those at high risk of advanced liver disease, who may previously have been missed.

They are not nuanced and are not intended to be restrictive: they do not supersede good clinical judgement. If you would like any advice regarding these topics, please contact Dr Jeremy Cobbold or Dr Jane Collier on 01865 2287 46/47/50, or by email Jeremy.cobbold@ouh.nhs.uk or jane.collier@ouh.nhs.uk

Hepatology Red Flags

Please investigate urgently and consider early Hepatology referral for patients with the following:

- Jaundice
- Hepatomegaly
- Splenomegaly
- Thrombocytopaenia
- Prolonged PT, low albumin in the context of abnormal LFTs

A. Mildly elevated Alanine Aminotransferase (ALT <4x ULN, +/- abnormalities in other LFTs)

1. Lifestyle history and intervention
    a. Alcohol history and abstinence
    b. Metabolic syndrome components and weight loss
    c. Stop potentially hepatotoxic medications (NB Herbal medications, steroids and OTC medications)
    d. Recheck LFTs *and AST* 3 months after intervention
        i. If normal → **consolidate lifestyle advice and recheck in 1 year**
        ii. If still abnormal,
2. Please arrange full chronic liver disease screen, including:
        i. **US abdomen**
        ii. **Viral** (Hep B S Ag, Hep C Ab)

iii. **Autoimmune** (ANA; Immunoglobulins; Liver autoantibodies (anti mitochondrial, anti smooth muscle, anti liver kidney microsomal); coeliac serology (anti endomysial or tissue transglutaminase, TTG))

iv. **Metabolic** (Lipid profile; HbA1c; Ferritin; TSH; Caeruloplasmin (if age <40); alpha-1 antitrypsin)

b. If POSITIVE for viral or autoimmune markers →

refer Hepatology (Dr Collier, Dr Cobbold: Dr Collier for viral hepatitis)

c. If screen NEGATIVE and persistently elevated or rising ALP, need to exclude primary sclerosing cholangitis →

refer Hepatology (Dr Collier, Dr Cobbold)

d. If screen NEGATIVE for viral or autoimmune markers, with steatosis/increased echogenicity on US →

likely NAFLD, Go to A-3

3. Calculate NAFLD Fibrosis Score (www.nafldscore.com)

a. If "low risk" →

address cardiovascular risk and reassess NFS in 2 years

i. assess cardiovascular risk factors and modify as appropriate (note: NAFLD and mildly abnormal LFTs is not a contraindication to statin use)

ii. aim for 7-10% weight loss over 6-12 months

iii. recalculate NAFLD fibrosis score after 2 years

b. If "indeterminate" or "high risk" →

refer Metabolic Hepatology (Dr Cobbold)

Note: Moderately to severely elevated ALT (>4x ULN, with or without abnormalities in other LFTs)

1. As per mildly elevated ALT, but check liver screen directly and do not wait 3 months
2. Please do not hesitate to contact us for advice- we would be happy to see directly

B. Increased hepatic echogenicity or steatosis on ultrasound scanning

1. Check LFTs and AST

a. If ALT elevated → Go to **A**.

b. If ALT not elevated → assess alcohol intake, components of the metabolic syndrome and calculate NAFLD fibrosis score (if alcohol intake within limits)

i. if 3 or more components and/or NFS "indeterminate" or "high" risk
→ **refer Metabolic Hepatology (Dr Cobbold)**

ii. if 2 or fewer components and NFS "low risk" →

address cardiovascular risk and reassess in 1 year with repeat LFTs +AST

2. If irregular liver contour and/or splenomegaly, suggests cirrhosis →

please send chronic liver disease screen and refer directly to Hepatology

Note: components of the metabolic syndrome-

- Abdominal obesity
- Type 2 Diabetes or Impaired Fasting Glucose
- Hypertension (treated or untreated)
- Dyslipidaemia (low HDL or elevated fasting triglycerides, treated or untreated)

C. Isolated elevated gamma-Glutamyl Transferase (γGT)

Please note: due to lack of sensitivity and specificity, we do not recommend use of γGT for routine liver function testing, nor to indicate excessive alcohol intake.

1. Lifestyle history and intervention as per **A-1**
2. Recheck LFTs *and AST* 3 months after intervention and proceed as per **B-1**

D. Isolated elevated Alkaline Phosphatase (ALP)

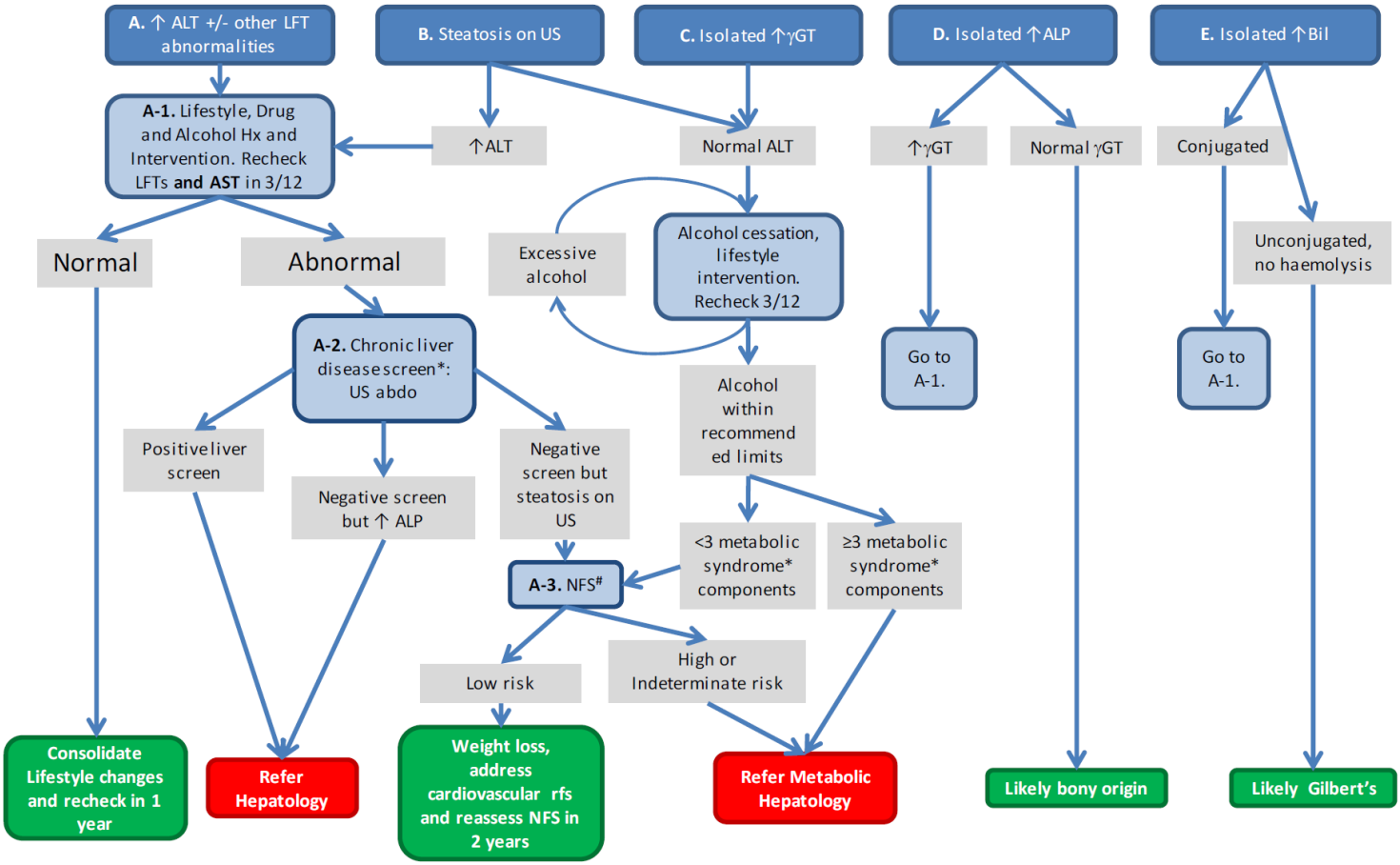
1. Check γGT
    a. If γGT normal → likely bone origin- please exclude pregnancy, check Bone profile and Vitamin D. Consider PTH testing and look for evidence of malignancy
    b. If γGT elevated → proceed as per **A-1**

E. Isolated elevated Bilirubin

1. Check conjugated/unconjugated ("split") Bilirubin
    a. If increased unconjugated Bilirubin (>20%) and haemolysis excluded →
    likely Gilbert's syndrome- reassure
    b. If conjugated hyperbilirubinaemia,
        i. look for and stop (if appropriate) potentially hepatotoxic medications then
        ii. proceed with abdominal US and chronic liver disease screen as per **A-1/A-2.**
        iii. If jaundiced ("red flag") please consider early referral.

Note on statins: there is no contraindication to statin use in chronic liver diseases if indicated. Statins are occasionally associated with a transaminitis, which is frequently a manifestation of NAFLD (*association is not causation!*). Statin-induced transaminitis is usually low-grade and not associated with clinical sequelae. If there is concern that a rise in LFTs (particularly ALT) is statin-related (i.e if ALT rises to 4x ULN from normal at baseline, or in association with myalgia), consider stopping the statin and proceeding as per guidance (section A).

Guidelines Summary: Incidental Finding of Abnormal Liver Function Tests



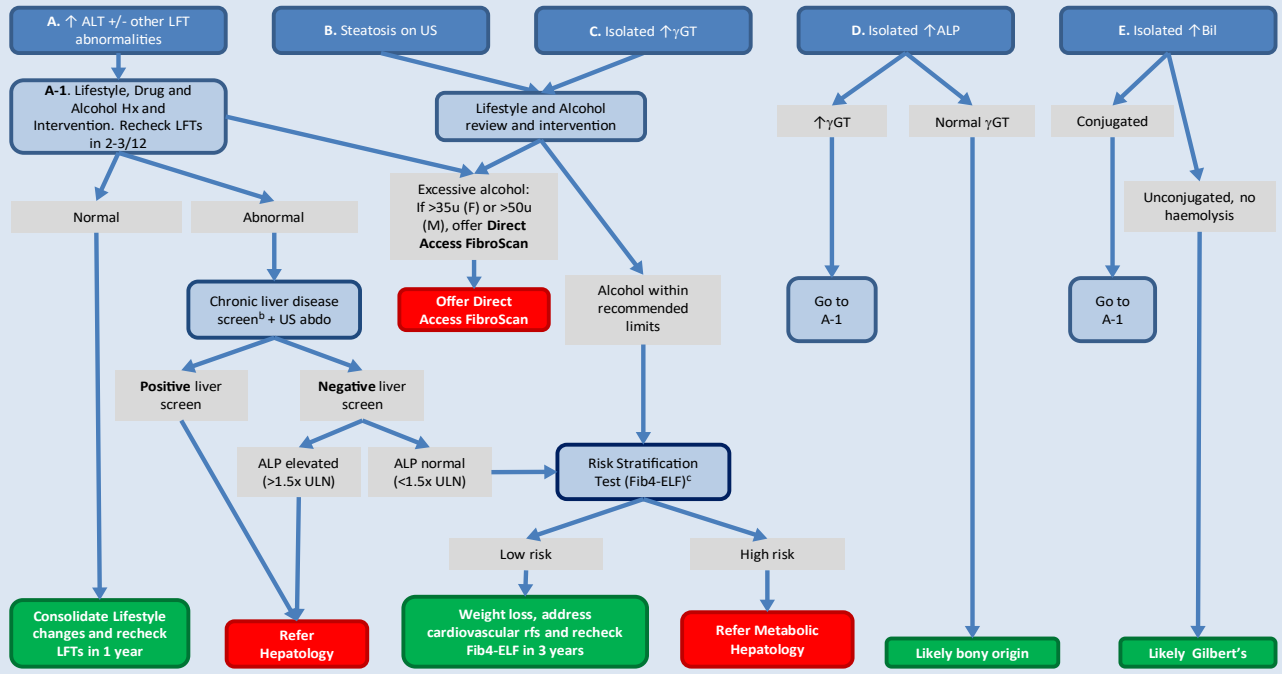
Red Flags: 1. Jaundice 2. Hepatomegaly/irregular liver 3. Splenomegaly 4. Low platelets 5. Low albumin/Prolonged PT

NFS = NAFLD Fibrosis Score- www.nafldscore.com

*For components of chronic liver disease screen and metabolic syndrome, please refer to text

Updated Metabolic Hepatology Clinic Referral Guidelines 2017 to date, Oxford University Hospitals NHS Trust and Oxfordshire CCG

Guidelines Summary: Investigating *Mildly* Abnormal Liver Tests[a]



Red Flags to trigger direct referral

1. Jaundice 2. Severe derangement in LFTs[a] 3. Hepatomegaly/irregular liver 4. Splenomegaly 5. Low platelets 6. Low albumin/prolonged PT

- [a]mildly elevated ALT: <4x ULN; moderate >4x <10x ULN; severe >10xULN. If moderate or severe, refer urgently direct to Hepatology
- [b]Chronic Liver Disease Screen consists of: Hep B sAg, Hep C ab, ANA, Liver autoantibodies, Immunoglobulins, Ferritin, HbA1c
- [c]Risk stratification test requested on ICE (search "Fib4" OR "ELF")
- If there are other queries, please use the new Hepatology email advice line: oxon.hepatologyadvice@nhs.net

Interpretation of Fib-4 and ELF test in NAFLD

Age <35	**ELF <9.0**		**Low risk for advanced liver fibrosis.** Treat metabolic risk factors and if there is ongoing risk suggest repeating test in 3 years.
	ELF >9.0		**High risk for advanced liver fibrosis.** Recommend referral to Metabolic Hepatology Clinic.
Age >35	**FIB-4 <1.3**		**Low risk for advanced liver fibrosis.** Treat metabolic risk factors and if there is ongoing risk, consider repeating FIB-4 in 3 years.
	FIB-4 1.3-2.67 (Indeterminate)	**ELF<9.5**	**Low risk for advanced liver fibrosis.** Treat metabolic risk factors and if there is ongoing risk consider repeating FIB-4 in 3 years.
		ELF>9.5	**High risk for advanced liver fibrosis.** Recommend referral to Metabolic Hepatology Clinic
	FIB-4 >2.67		**High risk for advanced liver fibrosis.** Recommend referral to Metabolic Hepatology Clinic.

If you have any queries regarding the interpretation of the results, if there are any atypical clinical features or hepatic comorbidity, please contact the Hepatology email advice line on oxon.hepatologyadvice@nhs.net

We like to offer all patients with NAFLD access to clinical trials. If your patient is interested then please email: Nellia.Sande@ouh.nhs.uk

Appendix 7.1, Table 1: Change in liver and cardio-metabolic health parameters from baseline to latest visit stratified by fibrosis stage (early and advanced fibrosis)

Measure Median (range)	Fibrosis Stage F0-F2 (Early Fibrosis)					Fibrosis Stage F3-F4 (Advanced Fibrosis)				
	N	Base.	Latest	Δ	*P* value	N	Base.	Latest	Δ	*P* value
Liver										
ALT, IU/L	50	66 (12-215)	48 (15-240)	-18	**0.0005**	37	50 (15-200)	40 (14-114)	-10	**0.014**
Transient elastography, kPa	30	8.4 (4.3-17.3)	7.3 (4.3-20)	-1.1	**0.009**	14	17.6 (6.8-75.0)	13.0 (6.1-57.0)	-4.6	0.22
Weight										
Weight, kg	48	97.7 (58.3-154.6)	96.8 (56.9-182.2)	-0.9	0.7	36	98.2 (70.0-159.5)	93.2 (71.9-166)	-5.0	**0.020**
Metabolic										
HbA_{1c}, mmol/mol	32	49 (34-124)	46 (30-110)	-3	0.27	28	48 (32-93)	47 (33-91)	-1	0.60
Total cholesterol, mmol/L	27	5.1 (2.50-9.0)	4.7 (2.70-8.1)	-0.4	0.33	20	4.0 (2.4-7.1)	3.7 (1.9-7.3)	-0.3	0.052
HDL, mmol/L	27	1.0 (0.5-1.7)	1.0 (0.6-1.9)	0.0	0.75	20	1.0 (0.7-2.4)	1 (0.7-1.9)	0	0.55
***CVD** (QRISK3)*										
Absolute risk, %	48	10.7 (0.1-42.1)	9.6 (0.1-37.8)	-1.1	0.53	36	16.0 (0.2-60.9)	15.4 (0.1-52.6)	-0.6	0.27

Appendix 7.1, Table 2: Comparison of baseline parameters in those who gained weight and those who lost weight between baseline and follow-up

Baseline Parameter Median (range)	Weight Gain	Weight Loss*	P value
n	58	101	
ALT, IU/L	55 (12-174))	51 (14-215)	1.0
n	38	70	
HbA1c, mmol/mol	45 (31-124)	53 (25-103)	0.16
n	30	44	
Tchol, mmol/L	4.7 (2.7-6.5)	4.5 (2.4-7.7)	0.85
n	58	101	
CVD QRISK3 Relative Risk	2.3 (0.8-11.1)	2.1 (0.7-11.5)	0.15
n	27	44	
Liver Stiffness, kPa	9.5 (4-75)	8.5 (3.5-36.3)	0.51

*or no change

n, number of patients with paired data;
Wilcoxon signed rank test between baseline and latest; bold *P* value represents statistical significance.

Appendix 7.1, Table 3: Change in metabolic and liver health variables from baseline to latest visit for patients with poorly controlled T2DM (HbA1c >58 mmol/mol)

Variable Median (range)	**N**	**Baseline visit**	**Latest visit**	**Δ**	***P* value**
Liver function test					
ALT, U/L	43	47 (21-189)	39 (11-98)	-8	**<0.0001**
AST, U/L	14	40 (21-171)	33 (24-84)	-7	0.1776
Weight					
Weight, kg	43	98.1 (55.0-143.7)	95.1 (53.9-180.5)	-3.0	**0.0002**
Metabolic					
HbA_{1c}, mmol/mol	43	76 (59-124)	62 (40-110)	-14	**<0.0001**
Total cholesterol, mmol/L	26	4.6 (2.7-7.7)	4.0 (1.9-8.1)	-0.6	**0.0012**
HDL, mmol/L	26	1.1 (0.5-1.7)	1.0 (0.60-1.6)	-0.1	0.8244
Triglyceride, mmol/L	18	3.27 (0.92-7.79)	2.34 (1.34 -8.98)	-0.93	0.2121
Systolic blood pressure, mmHg	37	141 (105-189)	133 (102-181)	-8.00	0.2802
Liver					
Fib-4 score	15	2.23 (0.52-7.26)	1.23 (0.42-9.26)	-1.00	0.7933
NFS	13	0.24 (-2.54-3.04)	-0.31 (-1.83-3.08)	-0.55	0.6975
Transient elastography, kPa	22	10.45 (4.4-36.3)	8.95 (4.4-30.4)	-1.5	0.0805
***CVD** (QRISK3)*					
Absolute Risk, %	41	17.8 (2.1-60.9)	18.6 (3.0-52.6)	0.8	0.9100
Relative Risk	41	2.7 (1.3-11.1)	2.6 (1.1-9.5)	-0.1	0.4390

Abbreviations: N, number of patients with paired data; Δ, difference between median at baseline and latest visit; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HbA_{1c}, glycated haemoglobin; HDL, high-density lipoprotein; CVD, cardiovascular disease.

Wilcoxon signed rank test between baseline and latest visit; bold *P* value represents statistical significance.

7.2 Appendix to chapter ‘Novel interventions for the treatment NALFD – a head to head comparison of GLP-1 analogues therapy and lifestyle intervention’

Figure 7.1: Subjects who achieved a 30% relative reduction in liver fat

There was no significant difference in the proportion of subjects who achieved a 30% relative reduction in liver fat (p=0.24) (Figure 7.1).

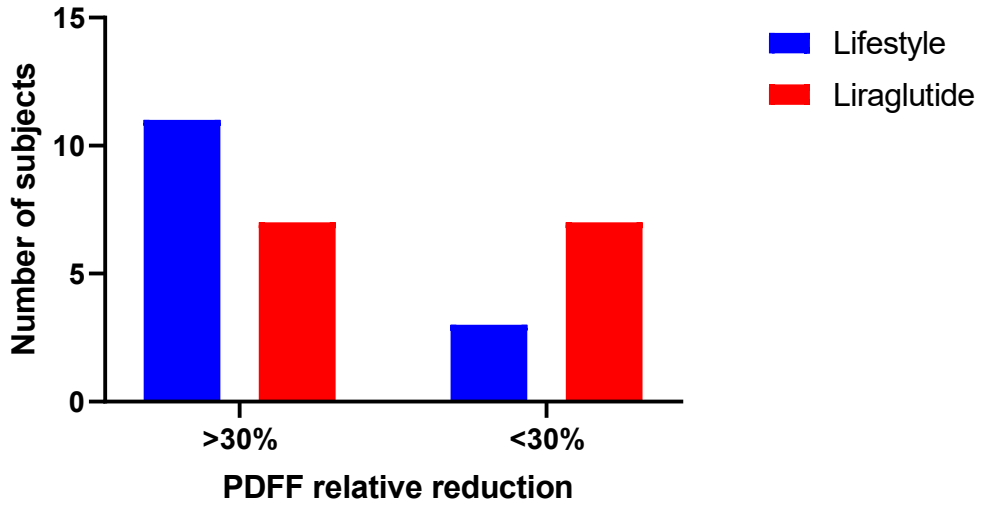
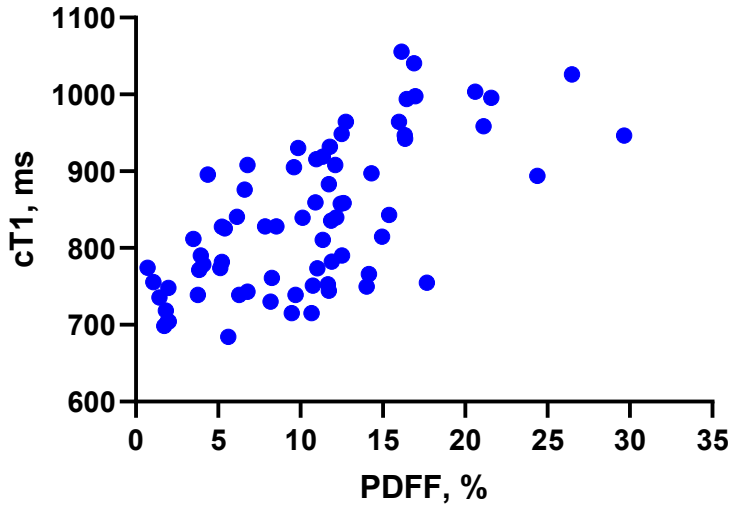


Figure 7.2: Correlation of liver fat (PDFF) with cT1

To investigate the relationship between liver fat and cT1, an analysis was performed on multiparametric data from all subjects and all scans collated as part of the LiLi Study irrespective of study intervention who had that had both PDFF and cT1 values recorded. A correlation was found between liver fat and cT1: r^2=0.44, p=<0.0001.



To investigate the primary drivers of improvement in MRI liver fat in all subjects in the LiLi Study irrespective of study intervention, weight was the only factor identified which governed the reduction in liver fat observed (Figure 7.3).

Figure 7.3: Weight contributes to change in liver fat

- Mixed effects model used on all paired data (n=72).

Covariate	For PDFF Linear coefficient (SE) [p-value]		
	Model 1	Model 2	Model 3
Weight	0.17 (0.049) **[0.0012]**	0.18 (0.05) **[0.005]**	0.18 (0.05) **[0.001]**
Age		0.01 (0.09) [0.9026]	0.003 (0.08) [0.9725]
Male gender		-4.01 (2.35) [0.0998]	-4.03 (2.29) [0.0903]
Lifestyle group			-2.11 (2.12) [0.3301]

- Adding multiple covariates to model identifies consistently that weight is significantly impacting change to fat. Age, gender and lifestyle group do not.

Analysis performed and figure provided by Perspectum Diagnostics

Furthermore, in a similar analysis of improvement in cT1 as a marker or liver inflammation and fibrosis in the LiLi study, again irrespective of study intervention, weight was found to be the most important factor governing cT1 improvement (Figure 7.4).

Figure 7.4: Weight contributes to change in cT1

- Mixed effects model used on all paired data (n=72).

Covariate	For cT1 Linear coefficient (SE) [p-value]		
	Model 1	Model 2	Model 3
Weight	2.8 (0.63) **[<0.0001]**	2.56 (0.63) **[0.0002]**	2.5 (0.63) **[0.0003]**
Age		-2.25 (1.1) [0.053]	-2.28 (1.1) [**0.049**]
Male gender		-36.7 (29.15) [0.2192]	-37.17 (29.11) [0.2135]
Lifestyle group			-16.35 (27.07) [0.5513]

- Adding multiple covariates to model identifies consistently that weight is significantly impacting change to cT1; age has a small but significant effect. Gender and lifestyle group do not.

Analysis performed and figure provided by Perspectum Diagnostics

Chapter 8 Published papers

8.1 Non-invasive markers of liver fibrosis: adjuncts or alternatives to liver biopsy?

frontiers in Pharmacology

REVIEW
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Non-invasive Markers of Liver Fibrosis: Adjuncts or Alternatives to Liver Biopsy?

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Liver fibrosis reflects sustained liver injury often from multiple, simultaneous factors. Whilst the presence of mild fibrosis on biopsy can be a reassuring finding, the identification of advanced fibrosis is critical to the management of patients with chronic liver disease. This necessity has lead to a reliance on liver biopsy which itself is an imperfect test and poorly accepted by patients. The development of robust tools to non-invasively assess liver fibrosis has dramatically enhanced clinical decision making in patients with chronic liver disease, allowing a rapid and informed judgment of disease stage and prognosis. Should a liver biopsy be required, the appropriateness is clearer and the diagnostic yield is greater with the use of these adjuncts. While a number of non-invasive liver fibrosis markers are now used in routine practice, a steady stream of innovative approaches exists. With improvement in the reliability, reproducibility and feasibility of these markers, their potential role in disease management is increasing. Moreover, their adoption into clinical trials as outcome measures reflects their validity and dynamic nature. This review will summarize and appraise the current and novel non-invasive markers of liver fibrosis, both blood and imaging based, and look at their prospective application in everyday clinical care.

Keywords: hepatology, fibrosis, biomarkers, elastography, MRI

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INTRODUCTION

As the gateway to the systemic circulation from the gut, the liver performs several critical functions, coordinating substrate metabolism, and detoxification as well as responding to immune stimuli. Its unique position and vascular supply also leaves it constantly exposed to potential injury, and liver damage, induced by hepatotoxins such as viral hepatitis, alcohol or excess fat, leads to a cycle inflammation and fibrosis. This process involves the deposition of extracellular matrix (ECM), which if degraded results in repair and regeneration; however should the source of liver injury persist, fibrosis may progress, and ultimately evolve to cirrhosis or end-stage liver disease.

Historically, the role of liver biopsy was both for diagnostic and staging purposes. Given the ability to diagnose the majority of liver conditions with blood testing alone, liver biopsy now primarily serves to quantify fibrosis in order to stage disease. This informs the clinician and patient regarding the urgency of initiating therapy (where possible) and prognosis. Indeed, recent longitudinal studies have demonstrated the presence and severity of fibrosis on biopsy as the single most important predictor of outcome in NAFLD, the commonest cause of liver disease in the

Western world, highlighting the need for disease staging for an individual patient (Angulo et al., 2015; Ekstedt et al., 2015).

Liver biopsy represents the "gold standard" for the assessment and quantification of liver fibrosis. However, its invasiveness engenders pain and significant potential complications, leading to poor patient acceptance (Cadranel et al., 2000). Other disadvantages include considerable cost (Pasha et al., 1998), and its inherent shortcomings related to sampling error and sample quality from needle biopsy specimens (Sherlock, 1962; Villeneuve et al., 1996; Bedossa et al., 2003; Merriman et al., 2006) have lead to questions regarding its suitability as the reference standard for liver fibrosis (Standish et al., 2006; Bedossa and Carrat, 2009). These factors, along with an acknowledgement of the need to stage disease without reliance on liver biopsy, have lead to an exponential interest in the identification and use of non-invasive markers of liver fibrosis.

The performance of a non-invasive marker of fibrosis is measured using liver biopsy staging of fibrosis as the reference standard. This is typically reported by sensitivity and sensitivity measurements, as well as an area under the receiver operating curve (AUROC) value, which indicates the ability of the marker (at different cut-offs) to correctly classify a patient into a particular category of fibrosis. Values of <0.7, $0.7-0.8$, $0.8-0.9$, and >0.9 indicate poor, fair, good, and excellent accuracy, respectively. Serum non-invasive tests can potentially achieve a perfect AUROC value, having been developed and calibrated using liver biopsy as a reference. The same is not true for imaging-based modalities, who are unable to achieve excellent diagnostic accuracy for fibrosis, an important point to consider when interpreting studies (Tsochatzis et al., 2011a). Another issue relates to "spectrum bias," where different stages of liver fibrosis may be represented unequally within a candidate study, leading to potentially misleading results, highlighting the importance of independent validation of markers.

Non-invasive markers of fibrosis are being incorporated into the routine clinical care of patients with liver disease, although the field continues to evolve. The importance of these markers comes with the stark realization that standard liver biochemistry is of little value in determining the severity of liver fibrosis in primary care (Armstrong et al., 2012). Instead, simple scores based on blood test results can enable a tier system whereby primary care physicians can determine the need for specialist referral for patients (Sheron et al., 2012). With the availability of accurate non-invasive tests, the ability to screen large cohorts for significant liver disease is now becoming a possibility, allowing the assessment of the true burden of liver disease in the general population (Koehler et al., 2016). Moreover, as novel anti-fibrosis therapies enter clinical trials, robust non-invasive markers are crucial to allow effective trial design and obviate the need for multiple, invasive liver biopsies to assess efficacy.

In this review, the types and application of several blood-based non-invasive markers of liver fibrosis are described, followed by a thorough description of current ultrasound based elastography methods and their utility in staging of liver disease. Finally, magnetic resonance imaging techniques to improve staging of liver fibrosis and cirrhosis are considered.

NON-INVASIVE SEROLOGICAL MARKERS

Whilst there have been many non-invasive biomarkers investigated and proposed, an ideal serological test for liver fibrosis which reduces the need to perform invasive investigations such as liver biopsy is still awaited. This ideal serological test would be quick to perform and analyse as well as inexpensive and reproducible. Furthermore, an ideal test would be able to distinguish between distinct entities in liver pathology such as inflammation and fibrosis, be unaffected by impairment in liver function, as well as being able to predict and track disease progression or regression.

When evaluating potential biomarkers, there is a need to ensure consistency in assessment and evaluation, and the related need for simple and robust classification systems (Veidal et al., 2010). This is important as the various serological biomarkers for liver fibrosis described in the medical literature to date have been appraised and validated in differing liver disease contexts, and so in different cohorts of patients. The majority of studies published have been performed in patients with hepatitis C (HCV), with fewer studies performed in patients with hepatitis B (HBV), non-alcoholic fatty liver disease (NAFLD), and alcoholic liver disease (ALD), with fewer still reported in rarer conditions such as in autoimmune liver diseases and hereditary haemochromatosis. As one may expect, these biomarkers perform differently in these differing liver disease contexts. As such, care must be taken to ensure that use of a specific biomarker test at individual patient level has both diagnostic and clinical validity in the context of the patient's illness (Balistreri, 2014). It is equally important to note that most of the biomarkers described in the literature work best in predicting established or advanced fibrosis when compared to the gold standard of liver biopsy, and that biomarkers generally do not perform well in indeterminate stages of liver disease.

To help aid understanding, non-invasive biomarkers of fibrosis may be broadly divided into two classes. Class I markers are direct serum markers reflecting ECM turnover and/or fibrogenic changes at cellular level in the liver. Class II markers of fibrosis are indirect serum markers based on algorithms and mathematical model of varying complexity and derived from changes that occur in liver function (Gressner et al., 2007; Veidal et al., 2010).

Class I (Direct) Biomarkers of Liver Fibrosis

Class I biomarkers are direct markers of fibrosis and reflect the molecular pathogenesis and turnover of liver ECM. The main source of fibrosis in the liver is derived from hepatic stellate cells (HSCs) and myofibroblasts (Korner et al., 1996; Friedman, 2000; Rockey and Friedman, 2006; Gressner et al., 2007; Henderson et al., 2013). Injury to the liver leads to the production of cytokines and to the activation of HSCs initially causing liver inflammation, and then to potentially irreversible change and scarring within the ECM tissue architecture.

Class I markers may be subcategorized into enzymatic markers, collagen (and related) markers, Glycoproteins and matrix-metalloproteinase markers, and Glycosaminoglycan markers (**Table 1**; Guechot et al., 1996; Murawaki et al., 1999; Walsh et al., 1999; McHutchison et al., 2000; Gressner et al.,

2007). Of these various classes, collagen derived markers and glycosaminoglycans (hyaluronic acid) have proven to be the most widely utilized in the development of new fibrosis biomarkers. Few class I markers are used in routine clinical practice as they largely do not directly correlate with whole tissue or body function and so provide data of limited clinical value. As such, their turnaround time is higher, availability is limited to mainly research and specialist settings and investigational costs are generally increased when compared to class II markers. Another limitation is that class I markers are neither all liver derived nor liver specific and so serum measurements do not always directly relate to liver activity. For example, during infection, where acute systemic inflammation co-exists, these markers may also be affected and their absolute values can reflect both fibrinolytic as well as fibrogenic activity.

Class II (Indirect) Biomarkers of Liver Fibrosis

Class II biomarkers provide an indirect reflection of liver ECM activity and fibrosis through the measurement of liver function or injury. As these tests reflect alterations in hepatic function and often correlate with signs and symptoms of illness, they are well established and used routinely in clinical practice to both diagnose and to monitor liver disease.

The broad range of activities the liver performs includes thermostasis, energy balance, carbohydrate, fat and protein metabolism, haemocoagulation, iron and hemoglobin metabolism, immune balance, production of bile acids and hormones, and clearance of toxins (Pratt and Kaplan, 2000; Limdi and Hyde, 2003; Tan et al., 2012; Neuman et al., 2014). Therefore, the number of class II biomarkers that may be tested is extensive.

In daily clinical practice, serological testing for liver function may be broadly categorized into the following categories:

- **Liver enzymes**, including alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT)
- **Markers of synthetic function**, such as prothrombin time (PT/INR), bilirubin, haptoglobin, albumin, Apolipoprotein A1, α2-macroglobulin, ceruloplasmin, transferrin and hepcidin
- **Other indirectly measured markers**, such as platelet count, α1-antitrypsin, ferritin, and certain adipokines (adiponectin and leptin; Ding et al., 2005).

As one may expect, although class II markers are more readily available and generally more cost-effective to employ, as proxy markers for fibrosis, their results are not as reliable or accurate as class I markers (Adams et al., 2005). Serum levels of these markers may be affected by a wide range of factors. It is well recognized that ALT levels, commonly thought to indicate the severity of liver disease, are wholly unreliable as a predictor of fibrosis (McCormick et al., 1996) and often within normal range in individuals with advanced disease (Mofrad et al., 2003). Indeed, one recent paper demonstrated a 56% prevalence of NAFLD in 103 volunteers with type 2 diabetes who had normal plasma aminotransferase levels (Portillo-Sanchez et al., 2015).

Combination or Panel Non-invasive Biomarkers

Whilst individual biomarkers have value as described, the combination of various biomarkers—with varying degrees of complexity—can greatly improve the sensitivity and specificity of these tests. Simple combination tests broadly employ class II biomarkers to indirectly assess liver fibrosis. The simplest of these is the AST/ALT ratio. Afdhal and Nunes summarized a number of studies that assessed its value, with most of these studies undertaken in patients with HCV reporting sensitivities between 53–78% and specificities between 90 and 100% for detecting liver fibrosis (Afdhal and Nunes, 2004). Another simple test is the AST to platelet ratio index (APRI), which uses the platelet count in addition to AST to predict fibrosis, first described in 2003 by Wai and colleagues in patients with HCV (Wai et al., 2003). The APRI score has also been shown to have some value in patients with HBV and PBC, although not in ALD, probably due to the confounding effects of alcohol on platelet suppression (Lieber et al., 2006; Loaeza-del-Castillo et al., 2008; Umemura et al., 2015; Xiao et al., 2015). Other simple indirect combination markers include the PGA (prothrombin time, γ-glutamyl transferase and apolipoprotein A1) and PGAA (PGA plus α-2 macroglobulin) indices, Pohl score and Forn's Index (Imbert-Bismut et al., 2001; Pohl et al., 2001; Forns et al., 2002). Another simple combination panel is the NAFLD fibrosis score (NFS), an easily accessible

TABLE 1 | Examples of Class I (direct) serum non-invasive biomarkers for liver fibrosis.

	References		References
Metalloproteinases (MMPs)	Murawaki et al., 1999; Walsh et al., 1999	Transforming growth factor (TGF)-β	Flisiak et al., 2002
Tissue inhibitors of metalloproteinases (TIMPs)	Walsh et al., 1999	Microfibril-associated protein 4 (MFAP4)	Molleken et al., 2009
Hyaluronic acid (HA)	Guechot et al., 1996	N Glycans- profiles	Molleken et al., 2009
Laminin	Korner et al., 1996	Procollagen type III amino-terminal peptide (PIIINP)	Guechot et al., 1996
Chitinase-3-like protein 1 (CHI3L1 or YKL-40)	Berres et al., 2009		
Collagen IV, VI	Shahin et al., 1992; Yabu et al., 1994; George et al., 1999		

online score which can be used by general practitioners to triage patients with suspected NAFLD in order to determine the need for referral for specialist assessment (Angulo et al., 2007). The AUROC values, sensitivities, specificities, and predictive values of these tests where available for liver fibrosis are provided in **Table 2**.

More complex combination biomarkers involve a panel of tests or complex algorithms, some of which are patented in the assessment liver fibrosis. These include the Fibrotest/Fibrosure®, Fibrometer®, and Hepascore® (Imbert-Bismut et al., 2001; Adams et al., 2005; Cales et al., 2005). As these tests generally include some class I markers as well as class II markers (**Table 2**), they have enhanced diagnostic accuracy, however this comes at an increased cost and reduced test availability in some cases, while also demanding greater physician time and effort to calculate and subsequently to interpret. Nevertheless, as these panels continue to improve, they are gaining increased acceptance in clinical practice, and large longitudinal studies have demonstrated their ability to predict prognosis and liver-related outcomes. For example, the Fibrometer® and FIB-4 showed good ability to predict significant liver-related events [AUROC values of 0.88 (0.83–0.91) and 0.87 (0.81–0.92)] in 373 patients with HCV over a median 9.5 year follow up, better than that predicted by fibrosis grade (Boursier et al., 2014). The Enhanced Liver Fibrosis (ELF) test similarly demonstrated prognostic capabilities in 457 patients with chronic liver disease followed a median over 7 years to predict death and clinical outcomes (Parkes et al., 2010). Moreover, a number of studies have now shown the value of the simpler investigations such as the NFS, the FIB-4 and APRI not only in the assessment of liver fibrosis, but also in estimating prognosis (Vallet-Pichard et al., 2007; Treeprasertsuk et al., 2013; Vergniol et al., 2014; Xiao et al., 2015). These findings are important considerations for the application of non-invasive markers with well-validated diagnostic and prognostic ability as outcome measures in clinical trials, obviating the need for repeated liver biopsies thereby enhancing recruitment and study efficiency. In routine clinical practice, the use of these scores alone or in combination can also significantly reduce the need for a liver biopsy, increasing the appropriateness and diagnostic yield from the remaining biopsies that are performed. For instance, Sebastiani and co-workers found that the combination of the APRI test with the Fibrotest-Fibrosure in 2035 patients with chronic HCV infection reduced the need for liver biopsy in 50–80% of cases, with high (>90%) diagnostic accuracy for significant fibrosis and cirrhosis (Sebastiani et al., 2009).

In summary, the quest to find an ideal non-invasive serological biomarker or combination of markers to diagnose and predict the outcome of liver fibrosis is ongoing, with considerable progress made over the past decade. Nevertheless, these investigations remain imperfect, particularly for the detection of intermediate fibrosis grades, and require judicious use tailored to the individual case, and detailed understanding to ensure their use is valid. As these investigations continue to be refined and improved, the need for staging liver biopsy will continue to decline over time.

NON-INVASIVE ULTRASOUND ELASTOGRAPHY

Ultrasound Based Elastography for the Assessment of Liver Fibrosis

Ultrasound based elastography has revolutionized the assessment of liver fibrosis over the last decade. The concept of elastography for the assessment of fibrosis is simple and stems from applying an external force to soft tissue, compressing the tissue, and thereby generating a shear wave which can be measured (Ophir et al., 1991). The speed of the shear wave propagating through the tissue corresponds to the tissue elasticity based on Young's modulus and hence, the amount of tissue fibrosis (Sandrin et al., 2003).

To date, a number of different types of ultrasound based elastography methods have been developed to assess liver fibrosis and diagnose cirrhosis (Thiele et al., 2015). One can broadly distinguish these elastography methods into two main groups: one-dimensional ultrasound elastography—transient elastography (TE); or two-dimensional (or B-mode) ultrasound which utilizes conventional ultrasound imaging - acoustic radiation force impulse (ARFI) imaging or point shear wave elastography (pSWE), real-time elastography (RTE), and real-time 2D shear wave elastography (2D-SWE). It is likely that some of these ultrasound-based elastography instruments will improve in time with the advent of novel technology.

Elastography measures tissue stiffness. Assessing liver fibrosis with ultrasound-based elastography only measures a small component of tissue stiffness. Many other factors can affect liver tissue stiffness and have been shown to affect the accuracy of assessing liver fibrosis with elastography. These factors were initially studied in TE and include: Inflammation from acute hepatitis (Arena et al., 2008a; Oliveri et al., 2008; Sagir et al., 2008); cholestasis from biliary tract obstruction (Millonig et al., 2008); blood congestion due to hepatic outflow obstruction and portal hypertension(Millonig et al., 2010; Shi et al., 2013); and food intake (Mederacke et al., 2009; Arena et al., 2013; Berzigotti et al., 2013) Another example that elastography measures more than just tissue fibrosis is the utilization of elastography to measure spleen stiffness in patients with cirrhosis; where spleen stiffness correlates with hepatic venous pressure gradient (HVPG; Colecchia et al., 2012; Sharma et al., 2013; Takuma et al., 2013) and reduces after liver transplantation (Chin et al., 2015). Although not studied extensively in other ultrasound-based elastography methods, all of these factors are thought to apply when interpreting liver stiffness measurements obtained with newer techniques (Bota et al., 2013a; Popescu et al., 2013).

Transient Elastography

Transient elastography (Fibroscan®, Echosens, Paris) is the first ultrasound-based elastography developed and is now a well-established non-invasive method of diagnosing and staging hepatic fibrosis (Sandrin et al., 2003). TE utilizes a mono-dimensional ultrasound to determine liver stiffness by measuring the velocity of low frequency elastic shear waves propagating through the liver. TE can be performed in a short time (typically

TABLE 2 | Combination scores of non-invasive serum biomarkers of liver fibrosis.

Index	Parameters	Year	Disease	*n*	AUROC for advanced fibrosis	Sens.	Spec.	NPV	PPV	Lead author
AST/ALT Ratio (De Ritis /Sheth)	AST/ALT	1998	HCV	139	–	53+	100+	81+	100+	Sheth et al., 1998
		2010	NAFLD	145	0.83	74	78	93	44	McPherson et al., 2010
		2015	PBC	137	0.59	–	–	–	–	Umemura et al., 2015
		2000	HCV	151	–	47+	96+	88+	74+	Park et al., 2000
BARD	BMI, AST, ALT, DM	2008	NAFLD	827	0.81	–	–	–	–	Harrison et al., 2008
		2010	NAFLD	145	0.77	89	44	95	27	McPherson et al., 2010
		2011	NAFLD	138	0.67	51	77	81	45	Ruffillo et al., 2011
		2016	NAFLD^	1038	0.76	74	66	–	–	Sun et al., 2016^
Bonacini-index	ALT/AST-ratio, INR, platelet count	1997	HCV	79	–	46	98	–	–	Bonacini et al., 1997
FIB-4	Platelet count, AST, ALT, age	2006	HCV/HIV	830	0.74–0.77	67	71	89	38	Sterling et al., 2006
		2010	NAFLD	145	0.86	85	65	95	36	McPherson et al., 2010
		2009	NAFLD	541	0.80	52	90	–	–	Shah et al., 2009
		2016	NAFLD^	1038	0.85	84	69	–	–	Sun et al., 2016^
		2015	PBC	137	0.71	–	–	–	–	Umemura et al., 2015
Fibrometer test	Platelet count, prothrombin index, AST, α2-macro-globulin, hyaluronic acid, urea, age	2005	HCV/HBV	383	0.89**	81**	84**	77**	86**	Cales et al., 2005
Fibrometer A	Prothrombin index,' α2 macroglobulin, hyaluronic acid, age	2005	ALD	95	0.96**	92**	93**	83**	97**	Cales et al., 2005
		2008	ALD	103	0.88	–	–	–	–	Nguyen-Khac et al., 2008
		2009	ALD	218	0.83	–	–	–	–	Naveau et al., 2009
Fibrotest (FT)	Haptoglobin, α2-macro-globulin, apolipoprotein A1. GGT, bilirubin, age, gender	2001	HCV	339	0.87	75	85	80	80	Imbert-Bismut et al., 2001
		2014	HBV^	2494	0.84**	61**	80**	–	–	Salkic et al., 2014^
		2009	ALD	218	0.83	–	–	–	–	Naveau et al., 2009
		2008	ALD	103	0.8	–	–	–	–	Nguyen-Khac et al., 2008
		2006	NAFLD	267	0.81	92	71	98	33	Ratziu et al., 2006
		2003	HCV	352	0.73	–	–	–	–	Poynard et al., 2003
Forns-index	Age, platelet count, GGT, cholesterol	2002	HCV	476	0.81–0.86**	94**	51**	96**	40**	Forns et al., 2002
Hepascore	Bilirubin, GGT, Hyaluronic acid, α2-macroglobulin, age, gender	2005	HCV	221	0.9–0.96	74–81	88–95	95–98	–	Adams et al., 2005
		2009	ALD	218	0.83	–	–	–	–	Naveau et al., 2009
		2008	ALD	103	0.83	–	–	–	–	Nguyen-Khac et al., 2008

(Continued)

TABLE 2 | Continued

Index	Parameters	Year	Disease	*n*	AUROC for advanced fibrosis	Sens.	Spec.	NPV	PPV	Lead author
Hui	Body mass index (BMI), platelet count, serum albumin, and total bilirubin	2005	HBV	235	0.79	88	50	92	38	Hui et al., 2005
Leroy-score	PIIINP, MMP-1	2004	HCV	194	0.88	58	92	–	91	Leroy et al., 2004
NAFLD Fibrosis Score (NFS)	Age, BMI, platelets, albumin, AST/ALT, IFG /diabetes	2007	NAFLD	733	0.82–0.88	77–82	71–77	88–93	52–56	Angulo et al., 2007
		2010	NAFLD	145	0.81	33–78	58–98	86–92	30–79	McPherson et al., 2010
		2016	NAFLD^	1038	0.84	77	70	–	–	Sun et al., 2016^
Fibrospect-II	Hyaluronic acid, TIMP-1, α2-macroglobulin	2004	HCV	696	0.82–0.83**	77–83**	66–73**	–	74**	Patel et al., 2004
PGA-index	Prothrombin time, GGT, apolipoprotein A1									
		1993	Mixed	169	–	91+	81+	–	–	Teare et al., 1993
		2008	ALD	103	0.84	–	–	–	–	Nguyen-Khac et al., 2008
PGAA-index	Prothrombin time, GGT, apolipoprotein A1, α2-macroglobulin	1994	ALD	525	–	79+	89+	–	–	Naveau et al., 1994
		2008	ALD	103	0.86	–	–	–	–	Nguyen-Khac et al., 2008
Pohl score	AST/ALT-ratio, platelet count	2001	HCV	221	–	41	99	93	85	Pohl et al., 2001
ELF score	Hyaluronic acid, TIMP-1, age, MMP-3	2004	HCV	496	0.77	95	29	95	28	Rosenberg et al., 2004
		2004	NAFLD	61	0.87	89	96	98	80	
		2004	ALD	61	0.94	93	100	86	100	
		2008	NAFLD	192	0.90	80	90	94	71	Guha et al., 2008
		2008	PBC	161	0.75	–	–	–	–	Mayo et al., 2008
		2014	Mixed^	1645	0.87	78	76	–	–	Xie et al., 2014^
SHASTA	HA, AST, albumin	2005	HCV-HIV	95	0.87	50	94	83	76	Kelleher et al., 2005
Fibrosis probability-index, FPI	Age, AST, cholesterol, insulin resistance (HOMA), past alcohol intake	2004	HCV	302	0.77–0.84**	96**	44**	93**	61**	Sud et al., 2004
APRI score	AST, platelet count	2003	HCV	270	0.8–0.88**	41–91**	47–95**	61–88**	64–86**	Wai et al., 2003
		2010	NAFLD	145	0.67	27	89	84	37	McPherson et al., 2010
		2008	ALD	103	0.43	–	–	–	–	Nguyen-Khac et al., 2008
		2015	PBC	137	0.84	–	–	–	–	Umemura et al., 2015
		2008	HBV	264	0.86**	87**	66**	81**	74**	Shin et al., 2008
Zeng	alpha2-macroglobulin, age, gamma glutamyl transpeptidase, and hyaluronic acid	2005	HBV	372	0.77–0.84**	35–95**	44–95**	51–86**	70–91**	Zeng et al., 2005

ALT, alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio; GGT, γ-glutamyltransferase; PIIINP, N-terminal propeptide of type III procollagen; TIMP, tissue inhibitors of metalloproteinases; MMP, matrix metalloproteinases.

***Values are for prediction of significant fibrosis.*

+Values are for prediction of cirrhosis.

^Metaanalysis.

less than 5 min) and has excellent intra- and inter- observer variability (Fraquelli et al., 2007; Boursier et al., 2008).

The Fibroscan® probe consists of a 3.5 MHz ultrasound transducer installed on the axis of a low amplitude vibrator (frequency of 50 Hz and amplitude of 2 mm peak-to-peak). To obtain liver stiffness measurements, the tip of the ultrasound transducer is placed in the right intercostal area, at the level of the right lobe of liver. When activated, the vibrator generates an elastic shear wave to the liver while the ultrasound transducer performed a series of ultrasound acquisitions (transmission/reception) with a repeat frequency of 4 kHz. A median value of at least 10 successful measurements with an Interquartile range (IQR) of ≤30% from the median and success rate of ≥60%, were considered as reflective of the liver stiffness or shear modulus of the liver. This value is expressed in kilopascals (kPa).

TE has been extensively studied for the assessment of fibrosis in many chronic liver diseases, particularly viral hepatitis. The two index studies on TE correlated liver stiffness with the degree of fibrosis (METAVIR staging) on liver biopsy due to chronic HCV infection (Castéra et al., 2005; Ziol et al., 2005). Since then, a number of comparable studies in HCV validated this finding (Coco et al., 2007; Arena et al., 2008b; Lupsor et al., 2009; Wang et al., 2009; Degos et al., 2010; Zarski et al., 2012; Afdhal et al., 2015), and a meta-analysis had reported excellent diagnostic accuracy for HCV-related cirrhosis with an AUROC of 0.95 (0.87–0.99; Shaheen et al., 2007). Similar studies were performed for patients with chronic HBV (Oliveri et al., 2008; Chan et al., 2009; Kim et al., 2009; Marcellin et al., 2009; Wang et al., 2009; Degos et al., 2010; Sporea et al., 2010; Cardoso et al., 2012; Goyal et al., 2013; Afdhal et al., 2015) and co-infection with HCV/HIV (de Lédinghen et al., 2006; Vergara et al., 2007; Kirk et al., 2009; Sánchez-Conde et al., 2010; Castera et al., 2014), with good correlation between liver stiffness and fibrosis. Furthermore, liver stiffness by TE has been shown to predict liver fibrosis in ALD (Nahon et al., 2008; Nguyen-Khac et al., 2008), NAFLD (Nobili et al., 2008; Yoneda et al., 2008; Lupsor et al., 2010; Wong et al., 2010a; Petta et al., 2011) and chronic cholangitides (notably, PBC and PSC; Corpechot et al., 2006, 2012). The diagnostic performance of TE have been confirmed by several meta-analyses to show excellent diagnostic accuracy for cirrhosis of (>90%), but only good accuracy for fibrosis (>80%; Shaheen et al., 2007; Friedrich-Rust et al., 2008; Stebbing et al., 2010; Tsochatzis et al., 2011b; Chon et al., 2012; **Table 3**).

Point Shear Wave Elastography/Acoustic Radiation Force Impulse Imaging

pSWE (Elastography point quantification, ElastPQ™, Phillips) and ARFI imaging (Virtual touch tissue quantification™, Siemens) are ultrasound-based elastography techniques which utilize B-mode ultrasound. This elastography technique can be incorporated into conventional ultrasound platforms, such as the iU22xMATRIX by Philips (Amsterdam, Netherlands) and Acuson S2000/3000 by Siemens Healthcare (Erlangen, Germany). Hitachi Aloka (Tokyo, Japan) also recently announced their pSWE method known as Shear Wave Measurement in the Radiological Society of North America

TABLE 3 | Diagnostic performance for ultrasound-based elastography methods for the detection of liver fibrosis.

Study	Aetiology	AUROC for $F > 2$ (95%CI)	Cutoff for F2	Sensitivity (%)	Specificity (%)	AUROC for F4 (95%CI)	Cutoff for F4	Sensitivity (%)	Specificity (%)
TE			**kPa**				**kPa**		
Talwalkar et al., 2007	All	0.87 (0.83–0.91)	–	70.0 (67.0–73.0)	84.0 (81.0–87.0)	0.96 (0.94–0.97)	–	87.0 (84.0–90.0)	91.0 (89.0–92.0)
Friedrich-Rust et al., 2008	All	0.84 (0.82–0.86)	7.7	–	–	0.94 (0.93–0.95)	13.0	–	–
Stebbing et al., 2010	All	–	7.7	71.9 (71.4–72.4)	82.4 (81.9–82.9)	–	15.1	84.5 (84.2–84.7)	94.7 (94.3–95.0)
Tsochatzis et al., 2011b	All	–	–	79.0 (74.0–82.0)	78.0 (72.0–83.0)	–	–	83.0 (79.0–86.0)	89.0 (87.0–91.0)
Shaheen et al., 2007	HCV	0.83 (0.03–1.00)	8.0	64.0 (50.0–76.0)	87.0 (80.0–91.0)	0.95 (0.87–0.99)	12.5	85.6 (77.8–91.0)	93.2 (90.2–95.3)
Chon et al., 2012	HBV	0.859 (0.857–0.860)	7.9	74.3	78.3	0.929 (0.928–0.929)	11.7	84.6	81.5
pSWE/ARFI			**m/s**				**m/s**		
Friedrich-Rust et al., 2012	All	0.87	1.34	79.0	85.0	0.93	1.80	92.0	86.0
Nierhoff et al., 2013	All	0.84 (0.80–0.87)	1.35	–	–	0.91 (0.89–0.94)	1.87	–	–
Bota et al., 2013b	All	0.85 (0.82–0.88)	1.30	74.0 (66.0–80.0)	83.0 (75.0–89.0)	0.93 (0.91–0.95)	1.80	87.0 (79.0–92.0)	87.0 (81.0–91.0)
Guo et al., 2015	All	0.85	–	76.0 (73.0–78.0)	80.0 (77.0–83.0)	0.94	–	88.0 (84.0–91.0)	83.0 (81.0–84.0)
Liu et al., 2015	NAFLD	0.90 (0.84–0.96)	–	80.2 (75.8–84.2)	85.2 (80.8–89.0)	–	–	–	–

Diagnostic performance for transient elastography (TE) and point shear wave elastography/acoustic radiation force impulse (pSWE/ARFI) for $F > 2$ and F4 presented in a number of meta-analyses.

Meeting in November 2015. pSWE/ARFI measures the speed of shear waves generated by acoustic pulses that lead to localized displacements of liver tissue (Nightingale et al., 2002). Liver stiffness measurement with pSWE/ARFI is performed at the right lobe of the liver, through the intercostal space, at the same site as TE. After selecting a region of interest, at a depth of 2–8 cm with B-mode ultrasound, the shear-wave velocity is measured within the defined region by using ultrasound tracking beams laterally adjacent to the single push beam (Friedrich-Rust et al., 2009). Similar to TE, pSWE/ARFI have good intra- and inter-observer agreement, with an intra-class correlation coefficient of between 0.84 and 0.87 (Boursier and Cales, 2010; Bota et al., 2012).

pSWE/ARFI has been described in many studies to be comparable to TE (Friedrich-Rust et al., 2009; Lupsor et al., 2009; Yoneda et al., 2010; Ebinuma et al., 2011; Piscaglia et al., 2011; Rifai et al., 2011; Sporea et al., 2012). Like TE, pSWE/ARFI is able to diagnose cirrhosis more accurately than significant fibrosis. Several meta-analyses of pSWE/ARFI confirmed good diagnostic accuracy for significant fibrosis, with mean AUROC of 0.84 to 0.87 and excellent diagnostic accuracy for cirrhosis of 0.91–0.94 (Friedrich-Rust et al., 2012; Bota et al., 2013a; Nierhoff et al., 2013; Guo et al., 2015). Based on data from these meta-analyses, the suggested cut-off values for the diagnosis of significant fibrosis were 1.30–1.35 m/s and for cirrhosis, the values were 1.80–1.87 m/s. A recent meta-analysis of pSWE/ARFI in NAFLD reported good diagnostic accuracy of pSWE/ARFI in assessing significant fibrosis in patients with NAFLD (AUROC of 0.898; Liu et al., 2015; **Table 3**).

Real-Time Elastography

RTE is a qualitative assessment of liver stiffness. For RTE, strain elastography is provided either by the operator applying manual compression (Friedrich-Rust et al., 2008) or compression is applied by the transmitted heartbeat (Koizumi et al., 2011). The reflected ultrasound echoes between the states of compression can be computed to measure displacement within each location of the tissue. Hence, the harder the tissue, the lower the amount of displacement of reflected ultrasound echoes before and under compression. A number of quantitative methods for RTE have been described to improve the comparability of this elastography technique, which include the elastic ratio, elastic index, elasticity score, and Liver Fibrosis Index (Friedrich-Rust et al., 2007; Kanamoto et al., 2009; Tatsumi et al., 2010; Koizumi et al., 2011; Colombo et al., 2012; Ferraioli et al., 2012a; Fujimoto et al., 2013; Yada et al., 2013). The basis of these quantitative methods for RTE is to provide a ratio of stiffness or elasticity. When comparing RTE to other ultrasound elastography methods, a recent meta-analysis reported lower diagnostic accuracy with RTE for significant liver fibrosis and cirrhosis, compared to TE or ARFI (Kobayashi et al., 2015). The marked heterogeneity of RTE studies was reported as one of the main reasons for lower diagnostic performance of RTE.

Real-Time 2D Shear Wave Elastography

Real time 2D-SWE is a relatively new ultrasound elastography method, which combines the initiation of a radiation force in tissue with focused ultrasonic beams and acquisition of transiently propagating resultant shear waves in real-time with a high-frequency ultrasound imaging sequence (Muller et al., 2009). In 2D-SWE, a large color coded elastography map is generated by combining several shear waves over time with rapid ultrasound acquisition (Thiele et al., 2016). 2D-SWE is performed quite similarly to pSWE/ARFI. Both the size and location of the region of interest can be chosen by the operator. Although different to pSWE/ARFI in technology, 2D-SWE is similar to pSWE/ARFI for the clinical user as it is implemented on a commercial B-mode ultrasound platform (Aixplorer®, Supersonic Imaging, Aix en Provence, France).

Liver stiffness measured by 2D-SWE has been shown to correlate with the stages of hepatic fibrosis on liver biopsy in chronic HCV (Ferraioli et al., 2012b), chronic HBV (Leung et al., 2013; Zeng et al., 2014), and alcohol-related liver disease (Thiele et al., 2016). In mixed cohorts of patients with chronic liver disease, several studies have shown similar results (Cassinotto et al., 2014; Jeong et al., 2014; Bota et al., 2015; Deffieux et al., 2015; Gerber et al., 2015; Grgurevic et al., 2015; Samir et al., 2015). There is now increasing evidence that 2D-SWE is a comparable elastography method to TE and pSWE/ARFI (Bavu et al., 2011; Ferraioli et al., 2012a; Leung et al., 2013; Cassinotto et al., 2014; Bota et al., 2015; Gerber et al., 2015; Thiele et al., 2016), although no published meta-analysis confirming these findings exists, as yet.

Comparative Performance of Ultrasound Based Elastography

Based on data from published literature on elastography, the diagnostic performance for significant fibrosis and cirrhosis for TE, pSWE/ARFI and probably 2D-SWE are equivalent. In contrast, RTE has a slightly lower diagnostic accuracy and remains limited to centers with experience with this method (Kobayashi et al., 2015). The diagnostic accuracy (AUROC values) for significant fibrosis using TE, pSWE/ARFI and 2D-SWE ranges between 0.65–0.97, 0.77–0.94, and 0.82–0.99 respectively, with excellent results consistently seen for cirrhosis, ranging between 0.80–0.99, 0.87–0.99, and 0.87–0.99, respectively. One advantage of TE is that it can be performed at the point of care, whereas second-generation elastrography techniques tend to require operators trained in ultrasound, reducing accessibility.

The failure rate of TE has been reported to range from 2.7 to 3.1% in obtaining any measurement, and 11.6 to 15.8% in acquiring unreliable results (Castéra et al., 2010; Wong et al., 2011). For pSWE/ARFI (Bota et al., 2013b) and 2D-SWE (Leung et al., 2013; Poynard et al., 2013; Elkrief et al., 2015), the failure rate for acquiring reliable results has been reported to be significantly lower than TE. Liver stiffness measured with 2D-SWE can be expressed either in kPa at a wide range of values (2–150 kPa) or in the form of shear wave velocity, in m/s. One of the main limitations of ARFI is the narrow range of shear wave velocity measurements of 0.5–4.4 m/s, in contrast to the range of TE of 2.5–75 kPa. This is thought to limit the ability of ARFI to define discriminative cut-off values for certain stages of fibrosis.

Quality criteria for liver stiffness measurements have previously been emphasized for TE to provide consistent and reproducible results. These criteria are less clear for pSWE/ARFI and 2D-SWE. The manufacturer of TE recommends that at least 10 successful liver stiffness measurements are acquired with an IQR of ≤30% from the median, and a success rate of ≥60%. The median value is taken as representative of liver stiffness. For pSWE/ARFI, most studies emulate the quality criteria of TE, where 10 valid measurements are performed and the median value deemed as the representative measure. Unreliable results for pSWE/ARFI were defined as an IQR/liver stiffness of greater than 30%. In both TE and pSWE/ARFI, a single shear wave is emitted temporarily at a single frequency for each measure. Hence, maintaining these quality criteria is fundamental to consistent measure of liver stiffness.

For 2D-SWE, these quality criteria do not apply. In 2D-SWE, the ultrasound transducer emits a plurality of pulse waves at increasing depth, using a very wide frequency band ranging from 60 to 600 Hz (Bercoff et al., 2004; Muller et al., 2009). The 2D-SWE transducer synchronously evaluate the velocity of several shear wave fronts over a wide frequency range. A real-time color coded map of elasticity is generated and is superimposed on the standard B-mode image. By selecting a region of interest in the center of the color map, the calculated value is the average of many values within the area (Cassinotto et al., 2014). Therefore, the quality criteria for 2D-SWE are not identical. Although not defined, a number of 2D-SWE studies have suggested the following: standard deviation/median ratio <0.1–0.3 and depth of measurement <5.6 cm; stable viscoelasticity map for at least 3 s with a homogenous color in the region of interest of at least 15 mm; and 3 measurements with the mean calculated as representative or using the median when >6 measurements are acquired (Sporea et al., 2013; Yoon et al., 2014; Procopet et al., 2015; Thiele et al., 2016).

Similar to serum biomarkers, ultrasound-based elastography is excellent for the non-invasive diagnosis of cirrhosis but only modestly good for significant fibrosis. When comparing TE with serum biomarkers, a number of studies have shown that both TE and serum biomarkers have equivalent performance for detecting significant fibrosis in HCV (Castéra et al., 2005; Degos et al., 2010; Zarski et al., 2012). Conversely, TE performs better than serum biomarkers for detecting cirrhosis due to HCV, if TE measurement is possible (lower applicability of TE compared to serum biomarkers, 80 vs. 95%; Degos et al., 2010; Zarski et al., 2012). While algorithms combining TE with serum biomarkers improve diagnostic accuracy in assessing fibrosis, particularly in HCV patients (Wong et al., 2010b; Boursier et al., 2011, 2012; Zarski et al., 2012), this strategy do not increase the overall diagnostic accuracy for detecting cirrhosis (Castéra et al., 2009; Boursier et al., 2011; Zarski et al., 2012). Furthermore, the combination of TE with serum fibrosis biomarkers is often advocated to better inform the clinician, allowing an informed judgment regarding the need for liver biopsy, for example where concordance exists in extremes of fibrosis, or discordance in intermediate stages.

Several studies have demonstrated that liver stiffness measured by ultrasound based elastography could predict clinical hepatic decompensation, liver related complication and prognosis (de Lédinghen et al., 2006; Robic et al., 2011; Vergniol et al., 2011; Corpechot et al., 2012; Merchante et al., 2012; Pang et al., 2014). Although a number of reports have described good correlation between liver stiffness and HVPG (Vizzutti et al., 2007; Bureau et al., 2008; Lemoine et al., 2008), the correlation between clinically significant portal hypertension and liver stiffness becomes less significant for HVPG values of >12 mmHg (Vizzutti et al., 2007). One potential explanation is that as cirrhosis progresses, the mechanism for portal hypertension is thought to be less dependent on intrahepatic resistance, and more dependent of the resultant hyperdynamic circulation and splanchnic vasodilatation (Reiberger et al., 2012). At present, ultrasound based elastography cannot replace HVPG for evaluation of portal hypertension or upper gastrointestinal endoscopy for detecting oesophageal varices. However, TE is an invaluable tool to risk stratify patients with clinically significant portal hypertension and identify patients at risk of developing HCC (Latinoamericana, 2015).

NON-INVASIVE MAGNETIC RESONANCE IMAGING

Magnetic Resonance Techniques for Liver Fibrosis Assessment

Magnetic resonance (MR) imaging techniques are attractive tools for liver fibrosis assessment. In contrast to ultrasound techniques, MR allows deep penetration into tissues, and unlike computed tomography avoids ionizing radiation. Repeated assessments can therefore be performed, without safety concerns. Furthermore, MR can assess the whole liver, eliminating sampling errors and can provide additional information on anatomy. There is now extensive experience in the use of MR for the evaluation of diffuse liver disease due to liver fat or iron deposition. Both magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) were found to be more accurate than histological assessment by a pathologist or by morphometry. (Roldan-Valadez et al., 2010; Raptis et al., 2012) MR has been used to study the epidemiology of NAFLD (Browning et al., 2004; Szczepaniak et al., 2005; Wong et al., 2012) and more recently it has been used as a surrogate end-point in two NAFLD clinical trials (Le et al., 2012; Loomba et al., 2015). MR techniques are also regarded as the gold standard for quantification of liver iron as they have shown an excellent inverse association with biochemically quantified hepatic iron concentration (Gandon et al., 2004; St Pierre et al., 2005). More recently, MR techniques for the assessment of liver fibrosis have been developed and have produced some promising results.

Magnetic Resonance Elastography

Similar to ultrasound based elastography techniques, magnetic resonance elastography (MRE) can determine liver stiffness by analysis of mechanical waves propagating through the liver. The technique requires the positioning of an external passive driver on the patient's body adjacent to the liver. The passive driver is connected to a pneumatic active driver placed outside the scanner

room which generates the mechanical waves that are propagated through the liver.

MRE for Liver Fibrosis Evaluation

In a large prospective single center study of 96 patients with mixed liver disease aetiologies (63% HCV; 8% NAFLD), MRE had excellent diagnostic accuracy for the differentiation of all stages of fibrosis, with an area under the receiver operating characteristic curve (AUROC) for ≥F3 and F4 of 0.99 and 1.00 respectively. (Huwart et al., 2008) Similar results have been obtained in patients with chronic HBV and HCV, (Ichikawa et al., 2012; Venkatesh et al., 2014a) and MRE was also shown to be closely related to morphometric quantification of liver fibrosis ($r = 0.78$; $p < 0.001$; Venkatesh et al., 2014b). In a meta-analysis of 697 individual patient data from 12 studies, MRE was found to have a good diagnostic performance for all stages of liver disease (with excellent accuracy in advanced disease; AUROC for ≥F3 and F4 of 0.93 and 0.92, respectively; Singh et al., 2015). A separate meta-analysis of 13 studies containing 989 patients (but not individual patient data) found pooled AUROC of 0.96 and 0.98 for the diagnosis of fibrosis stage ≥F3 and F4 respectively (Su et al., 2014). Despite these promising results, some concerns still exist regarding the true diagnostic accuracy of MRE, as some of the studies suffer from spectrum bias due to underrepresentation of patients with intermediate stages of fibrosis. For example, the distribution of fibrosis stages in one study was: F0 ($n = 28$), F1 ($n = 12$), F2 ($n = 6$), F3 ($n = 6$), F4 ($n = 20$; Wang et al., 2011).

MRE for the Evaluation of NALFD

MRE was found to be useful in the assessment of fibrosis in patients with NAFLD, with AUROC 0.92 and 0.89 for the diagnosis of fibrosis stage ≥F3 and F4 respectively (Loomba et al., 2014). Mixed results have been reported for use of MRE in the diagnosis of non-alcoholic steatohepatitis (NASH). Animal and retrospective human studies (Salameh et al., 2009; Chen et al., 2011), have shown some promise, although in a prospective study, MRE only had a modest diagnostic accuracy for NASH (AUROC of 0.73; Loomba et al., 2014).

Predictive Value of MRE in Patients with Cirrhosis

A recent study examined the value of MRE in predicting clinical outcomes in 430 patients with cirrhosis, with follow up data on 167 patients whose cirrhosis decompensated during the study. The authors showed that liver stiffness (LS) measured by MRE was independently associated with the presence of decompensation at baseline. Furthermore, a liver stiffness ≥5.8 kPa was a significant risk for decompensation (hazard ratio 4.96; 95%CI 1.4-17.0) in patients who had compensated liver disease at baseline (Asrani et al., 2014).

Diffusion Weighted Imaging

Diffusion weighted imaging (DWI) is a magnetic resonance technique that quantifies the diffusion of water molecules in tissues, and this is quantified as the apparent diffusion coefficient (ADC). The rationale for using this technique to assess liver fibrosis is that the deposition of collagen fibers in the liver would inhibit water diffusion, therefore leading to a decrease in the ADC. There is now considerable experience with this imaging technique, and a meta-analysis of 10 studies reporting the performance of DWI compared to histology, reported pooled AUROCs of 0.86, 0.83, and 0.86 for the diagnosis of any fibrosis (F1-4), significant fibrosis (F2-4) and bridging fibrosis (F3-4), respectively (Wang et al., 2012). This technique has not seen widespread application as it has been demonstrated that confounding factors like steatosis and perfusion also affect the ADC (Luciani et al., 2008; Leitao et al., 2013). Furthermore, when compared with other MR biomarkers of liver fibrosis, DWI was inferior to MRE (Wang et al., 2012) and T_1 mapping (Cassinotto et al., 2015), and only equivalent to transient elastography and serum based biomarkers (Lewin et al., 2007).

T_1 Relaxometry

T_1 relaxation time is a physical property of atoms that varies according to their electrochemical environment. The T_1 relaxation time of hydrogen atoms in water molecules is longer that the T_1 relaxation time of hydrogen atoms in long hydrocarbon chains like fatty acids. Therefore measuring T_1 can provide information about tissue composition. The observation that T_1 differed between healthy and diseased livers was made in very early studies of the clinical applicability of MR in visceral organs (Smith et al., 1981; Doyle et al., 1982). Despite this initial promise and subsequent studies in humans that showed some accuracy in the diagnosis of cirrhosis, T_1 imaging was largely developed for the anatomical assessment of the liver and particularly for the evaluation of liver tumors.

The confounding effects of iron (The Clinical NMR Group, 1987; Hoad et al., 2015) and inflammation / oedema (Chamuleau et al., 1988) on liver T_1 measures have limited the application of this technique until recently. The current impetus to develop non-invasive assessments of liver disease, combined with enhanced MRI methodologies, have resulted in improvements in the performance of liver T_1 as a biomarker of fibrosis, with some exciting results. Examples of studies examining the role of T_1 relaxometry for the assessment of liver fibrosis are outlined in **Table 4**.

Combination of T_1 Relaxation Time with Other Non-invasive Biomarkers

In the study by Hoad et al. (2015) liver T_1 is proposed as a biomarker of liver inflammation, which together with liver T_2^* for liver iron quantification and the ELF panel for fibrosis (William et al., 2004), are combined in a decision tree to determine who should be referred for liver biopsy. This approach resulted in a sensitivity of 90%, specificity of 71%, positive predictive value of 72% and negative predictive value of 89% in identifying those with significant fibrosis or inflammation.

Dynamic Contrast Enhanced MRI

Dynamic contrast enhanced (DCE) MRI requires the intravenous injection of hepatocyte specific gadolinium contrast agents like gadoxetic acid (Gd-EOB-DTPA; Primovist©; Bayer, Berlin, Germany). This technique gives an estimate of liver function, and has been shown to differentiate between healthy controls and patients with cirrhosis, and between the cirrhosis severity

TABLE 4 | T_1 relaxometry for the assessment of liver disease.

Study	Study design	Patients	Main findings
Smith et al., 1981	HV vs. patients with diffuse liver disease	HV ($n = 15$) Cirrhosis ($n = 5$) CAH* ($n = 1$)	Longer T_1 in cirrhosis and CAH
Doyle et al., 1982	HV vs. patients with diffuse liver disease	HV ($n = 12$) Cirrhosis ($n = 10$)	Longer T_1 in cirrhosis
The Clinical NMR Group, 1987	T_1 vs. histology	Patients with suspected parenchymal disease ($n = 55$)	Tendency toward longer T_1 in cirrhosis/hepatitis
Thomsen et al., 1990	HV vs. disease controls vs. cirrhosis T_1 vs. histology in a subset	HV ($n = 7$) Disease controls ($n = 17$) Cirrhosis ($n = 15$) Histology subset($n = 10$)	Longer T_1 in cirrhosis No association with histology
Keevil et al., 1994	HV vs. patients with diffuse liver disease	HV ($n = 42$) Liver disease ($n = 44$)	Longer T_1 in patients with cirrhosis and CAH
Heye et al., 2012	HV vs. cirrhosis	HV ($n = 31$) Cirrhosis ($n = 61$)	T_1 longer in cirrhosis
Kim et al., 2012	Non cirrhotic patients vs. CHB cirrhosis	Non cirrhotic patients ($n = 92$) HBV cirrhosis ($n = 87$)	T_1 shorter in cirrhosis
Cassinotto et al., 2015	HV vs. cirrhosis	HV ($n = 40$) Cirrhosis ($n = 89$)	T_1 longer in cirrhosis
Hoad et al., 2015	T_1 vs. histology	Training cohort ($n = 64$) Validation cohort ($n = 46$)	AUROC for cirrhosis 0.92 AUROC for advanced fibrosis 0.81

HV, healthy volunteers; CAH, chronic active hepatitis; HBV, hepatitis B virus; AUROC, area under the receiver operating characteristic curve.
**Chronic active hepatitis is a historical term used to describe a hepatitis of unknown etiology, now believed to be chronic hepatitis C and is no longer used in clinical practice.*

assessed by Child-Pugh stage or MELD score (Haimerl et al., 2013, 2014; Nilsson et al., 2013; Verloh et al., 2014; Zhao et al., 2015). Retrospective studies have also shown good accuracy for the diagnosis of NASH (AUROC 0.85; Bastati et al., 2014), and for the assessment of fibrosis (Feier et al., 2013; Ding et al., 2015). Furthermore, in a study by Feier et al. fibrosis was the only independent histological predictor of the relative T_1 change on DCE MRI (Feier et al., 2013).

Multi-Parametric MR Protocols

Combinations of several techniques for liver fibrosis assessment have also been examined. In a retrospective study of patients who had undergone liver MR, the combination of parameters derived from DWI, DCE MRI and susceptibility weighed imaging resulted in improved diagnostic accuracy for the classification of fibrosis (AUROC for F0 vs. F1-4: 0.95; F0-1 vs. F2-4: 0.95; F0-2 vs. F3-4: 0.90; F0-3 vs. F4: 0.93; Feier et al., 2016). Incorporation of MR texture analysis techniques with other MR modalities has also produced some promising early results (House et al., 2015; Wu et al., 2015).

Iron Corrected T_1

Liver iron is one of the major confounders in the use of liver T_1 as a biomarker of liver fibrosis, as discussed above. One innovation that has led to improved diagnostic accuracy for liver T_1 was the development of the liver "iron corrected T_1" (cT_1), which removes the confounding effect of iron from the T_1 measurements making it much more widely applicable (Tunnicliffe et al., 2014). In a prospectively recruited cohort of patients undergoing liver biopsy for the assessment of fibrosis, cT_1 showed excellent diagnostic accuracy against the Ishak staging system of fibrosis with an AUROC of 0.94 for the diagnosis of patients with any degree of fibrosis (F0 vs. $F \geq 1$; Banerjee et al., 2014). Furthermore, liver cT1 was used to derive the liver inflammation and fibrosis (LIF) score, a standardized continuous (0–4) score recently shown to predict liver related

clinical outcomes, and is thus of potential use in predicting prognosis in patients with chronic liver disease (Pavlides et al., 2016).

Molecular MR Imaging

Molecular MR imaging represents a unique implementation of MR technology to visualize biological processes at the cellular and molecular level. Studies examining these technologies are still restricted to animal models and it remains to be seen whether they are safe and clinically applicable in humans.

Type I collagen is a major constituent of liver fibrosis. As it deposited in the extracellular space, it is an ideal target for molecular MR imaging. A molecular probe (EP-3533) to type I collagen has been developed, and is the most extensively studied probe in molecular MR imaging of liver fibrosis. When injected, this probe leads to shortening of the T_1 relaxation time. In a feasibility study (Polasek et al., 2012), animals with fibrosis were found to have greater signal intensity compared to controls (0.55 vs. 0.39; $p < 0.05$) when imaged after EP-3533 injection. Furthermore, EP-3533 content measured in the sacrificed animals had a strong correlation with Ishak staging ($r = 0.79$ to 0.84 depending on the animal model). A subsequent study demonstrated that EP-3533 was superior to other MR biomarkers of fibrosis (T_1 relaxation time, $T_1\rho$, DWI and magnetisation transfer techniques; Fuchs et al., 2013). In their latest study, the group used a clinical scanner and showed that the technique can be useful in monitoring therapeutic effects using the bile duct ligation animal model of fibrosis (Farrar et al., 2015). Control animals, or animals that responded to rapamycin developed less fibrosis and had lower enhancement after EP-3533 compared to animals that did not receive or did not respond to treatment.

CONCLUSION

In summary, this review serves to provide a reference for the application of both serum and imaging based non-invasive markers of liver fibrosis. The use of these important clinical adjuncts has been discussed in detail, although given the plethora of non-invasive markers reported in the literature, the authors have specifically focused on the most well-known and available tools. While serum based algorithms and established elastography methods are being validated in large clinical cohorts of different liver diseases and demonstrate important predictive power in detecting liver related outcomes, imaging methodologies evolve apace, with promising novel approaches giving simultaneous diagnostic, staging as well as prognostic information entering the non-invasive arena.

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JR devised, wrote and revised the manuscript, provided funding for open access. JC, MP, and AM wrote sections of the manuscript and critically reviewed the entire paper.

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The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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8.2 A multidisciplinary approach to the management of NAFLD is associated with improvement in markers of liver and cardio-metabolic health

ORIGINAL ARTICLE

A multidisciplinary approach to the management of NAFLD is associated with improvement in markers of liver and cardio-metabolic health

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a globally prevalent health problem, associated in its more severe forms with increased liver-related and cardiovascular-related morbidity and mortality. We established a multidisciplinary metabolic hepatology clinic in 2014 and have analysed the clinical data to evaluate the effectiveness of this service.

Patients with NAFLD (n=165) who had attended two or more appointments were included. Prespecified clinical data were collected prospectively at clinic appointments and analysed retrospectively. Interventions offered included lifestyle advice, signposting to weight loss services and pharmacological treatment of diabetes and cardiovascular risk factors.

Median follow-up was 13 months (range: 2–34). 59% (n=97) of patients had type 2 diabetes mellitus (T2DM). 53% (n=87) underwent liver biopsy of whom 18% (n=16) had cirrhosis. Median alanine aminotransferase (ALT) reduced by 11 IU/L (p<0.0001), median weight reduced by 3.3 kg (p=0.0005). There were significant reductions in HbA1c, total cholesterol and liver stiffness. Specifically, in patients with T2DM, HbA1c decreased by 4 mmol/mol (p=0.01) with significant reductions in ALT, weight and total cholesterol. Relative cardiovascular risk assessed by the QRISK3 score reduced in the whole cohort and in those with T2DM. Health economic modelling suggested the clinic intervention among those patients with poorly controlled T2DM was cost-effective.

In conclusion, a multidisciplinary approach to the management of patients with NAFLD in this observational cohort study was associated with improvements in liver-related and cardio-metabolic related health parameters and with evidence of cost-effectiveness in patients with poorly controlled T2DM.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a globally prevalent public health burden. Estimates indicate that between 20% and 30% of many populations are affected and the prevalence is expected to rise with increasing prevalence of obesity and type 2 diabetes mellitus (T2DM).[1]

NAFLD is considered a disease spectrum from hepatic steatosis, which may be accompanied by histologically defined inflammation (non-alcoholic steatohepatitis, NASH), with or without liver fibrosis (typically classified as mild fibrosis (stages: F0–F1), significant fibrosis (F2) or advanced fibrosis (stages: F3–F4) with F4 fibrosis being a diagnosis of cirrhosis).[2] Liver cirrhosis confers a significantly increased risk of hepatic decompensation and development of hepatocellular carcinoma. The presence of advanced fibrosis predicts reduced transplant-free survival, increased liver-related mortality and all-cause mortality.[3 4] Nevertheless, the principal causes of morbidity and mortality from NAFLD are from cardiovascular and metabolic complications.[3 4] Furthermore, T2DM is the strongest risk factor for NAFLD disease progression.[5] Those with both NAFLD and T2DM have accelerated liver disease progression and more prevalent and severe complications of diabetes.[6 7] Studies have suggested that even significant fibrosis is associated with increased mortality.[8]

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NAFLD should thus be considered a cardio-metabolic disease as well as a liver disease with implications for risk stratification and for management. Accordingly, appropriate disease staging at presentation will identify those at high risk of adverse outcomes who would benefit from more intensive management. As there are no currently licenced treatments specifically for NAFLD, management focuses on modification of risk factors for NAFLD progression and cardiovascular disease within a multidisciplinary framework.[9–11]

This study aimed to evaluate the impact of a metabolic hepatology clinic that adopts a multidisciplinary, holistic approach to treat NAFLD and uses a number of markers of liver and cardio-metabolic health to assess its performance. These included thorough assessment of surrogate markers of liver injury, diabetes control and cardiovascular risk. Furthermore, this study evaluated the effects of the clinic on quality adjusted life expectancy (QALE), and estimated the cost-effectiveness of the approach.

METHODS

Intervention: a multidisciplinary metabolic hepatology clinic

The Oxford University Hospitals National Health Service (NHS) Foundation Trust (OUH) delivers a weekly secondary/tertiary multidisciplinary metabolic hepatology clinic, managing patients from across Oxfordshire, UK, and the surrounding regions. Referrals to the clinic are received from both primary care and secondary care settings. OUH works closely with the Oxfordshire Clinical Commissioning Group and has produced guidelines[12] to assist primary care professionals to investigate and refer patients. These guidelines assist general practitioners to risk stratify patients and help ensure that those at high risk of advanced liver disease are referred to and managed in secondary care (this pathway was updated in November 2017 after the period covered by the analysis in this paper). From January 2015 to November 2017, local guidance recommended that patients with suspected NAFLD in primary care first underwent risk-stratification with the NAFLD fibrosis score (NFS) with referral of those patients with indeterminate or high-risk scores to the metabolic hepatology clinic. However, referrals without risk stratification were not refused and risk stratification was performed in clinic. If the diagnosis was unclear, patients were referred to the general hepatology clinic. Risk stratification would then be performed by NFS, Fib-4 score and/or FibroScan and subsequent appointments scheduled in the metabolic hepatology clinic if appropriate.

The clinic is jointly led by hepatologists and diabetologists/metabolic physicians. The aim of the clinic is to improve liver-related and cardiovascular health in patients with NAFLD/NASH. A range of lifestyle and medical interventions are employed. Emphasis is given to weight management and the achievement of meaningful weight loss in those who are overweight or obese via dietary and lifestyle modifications. Medications are used to aid reduction of cardiovascular risk, for example, the use of antihypertensive and statin therapy, as well as to improve diabetes control where appropriate. For the control of diabetes, therapies that are weight-neutral or encourage weight loss such as SGLT2 inhibitors and GLP-1 agonists are favoured.[13–16] These newer diabetes therapies are further beneficial as they also reduce cardiovascular risk.[17–19]

The clinic is supported by specialist nurses performing transient elastography (FibroScan) and anthropometrics immediately prior to the medical consultation and by specialist practitioners via the *Here for Health* service. This is a special service at OUH that bridges the link between the acute hospital setting and currently available community services. It provides a range of health and well-being advice including weight management (diet, lifestyle and exercise), but also smoking cessation, alcohol reduction and signposting to mental health services (www.ouh.nhs.uk/patient-guide/here-for-health/default.aspx). Blood testing, imaging, liver biopsy and screening for hepatocellular carcinoma in patients with established cirrhosis are performed where clinically appropriate and in accordance with relevant clinical guidelines. Patients with mild disease, either on non-invasive markers (low-risk NFS or Fib-4, FibroScan <8 kPa without adjustable metabolic complications, or liver biopsy F≤2 in absence of metabolic complications) are typically discharged from clinic. Exceptions include those with complex metabolic comorbidities, florid NASH in young people and others at the clinician's discretion. Local guidance since November 2017 recommends repeat non-invasive risk stratification in patients with mild disease in 3 years to look for evidence of progression.

Study analysis

A retrospective analysis of all patients who were managed through the metabolic hepatology clinic at OUH between inception in March 2014 until May 2017 was performed. Patients were included in the analysis if they had (1) attended the clinic at least twice, (2) had an alanine aminotransferase (ALT) level recorded at baseline and their latest clinic visit and (3) had weight recorded at baseline. Clinical data from all patients attending since clinic inception were collected on a clinic proforma and subsequently recorded on a centrally held, secure, departmental clinical spreadsheet for audit purposes.

Diagnosis of NAFLD was made according to the National Institute for Health and Care Excellence (NICE)[20] and other guidance; either radiologically (liver ultrasound or liver MRI), histologically on liver biopsy, or on the basis of persistently elevated liver enzymes (elevated ALT and/or aspartate aminotransferase (AST) levels (>40 IU/L) in the context of

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features of the metabolic syndrome and where other causes of liver pathology had been excluded. Patients with known hepatic comorbidity, patients who did not have a diagnosis of NAFLD, those who had type 1 diabetes and those who had previously undergone or who underwent bariatric surgery during the follow-up period were excluded from the analysis (online supplementary figure 1).

Patients underwent assessments of liver and cardio-metabolic health using routine non-invasive tools including measurement of serum ALT, AST and liver stiffness measurement by transient elastography

Table 1 Baseline characteristics of the total cohort and by T2DM status

Baseline characteristic, median (range) or number (%)	Total cohort n=165		T2DM subgroup n=97		Non-T2DM subgroup n=68	
Demographic						
Age, years	53	(16–79)	57	(29–79)	47.5	(16–69)
Sex, male	106	(64.2%)	62	(63.9%)	44	(64.7%)
Caucasian ethnicity	132	(80.0%)	78	(80.4%)	54	(79.4%)
Metabolic						
HbA1c, mmol/mol*	46	(25–124)	59	(35–124)	36.5	(25–46)
Total cholesterol, mmol/L††	4.4	(2.4–9.0)	4.0	(2.4–7.7)	4.9	(2.7–9.0)
HDL, mmol/L††	1.0	(0.5–2.4)	1.0	(0.5–1.7)	1.0	(0.8–2.4)
Triglyceride, mmol/L†	1.93	(0.52–17.01)	2.13	(0.52–10.71)	1.72	(0.68–17.01)
Systolic blood pressure, mm Hg‡	140	(105–190)	140	(105–189)	138	(108–190)
Metabolic syndrome (MetS)						
BMI, kg/m^2	33.3	(23.9–72.1)	34.0	(23.9–52.6)	32.5	(24.2–72.1)
Obesity (BMI>30 kg/m^2)	120	(72.7%)	71	(73.2%)	49	(72.1%)
Hypertension	93	(56.4%)	72	(74.2%)	21	(30.9%)
Dyslipidaemia	105	(63.6%)	71	(73.2%)	34	(50.0%)
Number of MetS components, /4	3	(0–4)	3	(1–4)	2	(0–3)
Lifestyle						
Alcohol, units/week	0	(0–21)	0	(0–21)	0	(0–20)
Current smoker	10	(6.1%)	6	(6.2%)	4	(5.9%)
Ex-smoker	53	(32.1%)	35	(36.1%)	18	(26.5%)
Liver						
ALT, IU/L	52	(12–215)	50	(12–200)	54	(12–215)
Abnormal ALT (>40 IU/L)	111	(67.3%)	60	(61.9%)	51	(75.0%)
Transient elastrography, kPa§	9.2	(3.5–75.0)	10.1	(4.3–75.0)	8.2	(3.5–53.3)
Biopsy characteristics						
Number (%)	87	(52.7%)	54	(55.7%)	33	(48.5%)
NAS, /8¶	5	(2–8)	5	(2–8)	5	(2–7)
NASH¶	57	(72.1%)	36	(73.5%)	21	(70.0%)
Fibrosis stage, (F0–F4)**	2	(0–4)	3	(0–4)	2	(1–4)
F0 (n, %)	1	(1.2%)	1	(1.9%)	0	(0.0%)
F1 (n, %)	15	(17.2%)	6	(11.1%)	9	(27.3%)
F2 (n, %)	34	(39.1%)	20	(37.0%)	14	(42.4%)
F3 (bridging fibrosis) (n, %)	21	(24.1%)	14	(25.9%)	7	(21.2%)
F4 (cirrhosis) (n, %)	16	(18.4%)	13	(24.1%)	3	(9.1%)

Biopsy characteristics provided for available data.
*Number of patients with baseline measurement, n=136, 88, 48 (of total cohort, T2DM subgroup, non-T2DM subgroup, respectively).
†N=100, 59, 41.
‡N=137, 82, 55.
§N=133, 73, 60.
¶NAS (NAFLD activity score) (/8); NASH defined as NAS>5; number of patients with measurement per group (percentage with NASH): n=79, 49, 30 in each column group.
**Fibrosis stage (Brunt's scale) (F0–F4); cirrhosis defined as fibrosis stage F4 or historical diagnosis in the case of one missing biopsy score. Number of patients with measurement per group (percentage): n=86, 53, 33.
††n=113, 68, 45.
ALT, alanine aminotransferase; BMI, body mass index; HDL, high-density lipoprotein; NAS, NAFLD activity scores; NASH, non-alcoholic steatohepatitis; T2DM, type 2 diabetes mellitus.

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Table 2 Change in liver and cardio-metabolic health parameters from baseline to latest visit

	Total cohort					T2DM subgroup				
Measure (median)	N	Base	Latest	Δ	P value	N	Base	Latest	Δ	P value
Liver function test										
ALT, IU/L	165	52 (12–215)	41 (11–240)	−11	**<0.0001**	97	50 (12–200)	40 (11–125)	−10.0	**<0.0001**
AST, IU/L	65	40 (15–171)	33 (14–105)	−7	**0.011**	35	35 (16–171)	31 (14–63)	−4.0	0.13
Weight										
Weight, kg	159	97.3 (55.0–206.0)	94.0 (53.9–182.2)	−3.3	**0.0005**	94	96.8 (55.0–154.6)	94.6 (53.9–180.5)	−2.2	**0.0030**
Metabolic										
HbA1c, mmol/mol	112	49 (25–124)	47 (22–110)	−1.5	**0.0045**	84	59 (35–124)	55 (33–110)	−4.0	**0.011**
Total cholesterol, mmol/L	76	4.7 (2.4–9.0)	4.0 (1.9–8.1)	−0.7	**0.0023**	48	4.1 (2.4–7.7)	3.9 (1.9–8.1)	−0.20	**0.0071**
HDL, mmol/L	76	1.0 (0.5–2.4)	1.0 (0.6–1.9)	0.0	0.75	48	1.0 (0.5–1.7)	1.0 (0.6–1.9)	0.00	0.85
Triglyceride, mmol/L	47	2.1 (0.67–17.0)	1.9 (0.6–9.0)	−0.25	0.28	29	2.1 (0.7–7.8)	2.0 (0.6–9.0)	−0.12	0.36
Systolic blood pressure, mm Hg	125	140 (105–189)	135 (102–193)	−5	0.24	74	141 (105–189)	136 (102–193)	−5	0.76
Liver										
Fib-4 score	62	1.1 (0.2–7.3)	1.2 (0.2–9.3)	0.05	0.71	35	1.5 (0.4–7.3)	1.2 (0.4–9.3)	−0.28	0.94
NFS	59	−1.0 (−6.5 to +5.8)	−0.69 (−6.0 to +3.9)	0.32	**0.0012**	33	0.02 (−3.9 to −3.0)	−0.3 (−4.7 to −3.1	−0.33	0.089
Transient elastography, kPa	73	9.1 (3.5–75)	7.8 (3.2–57)	−1.3	**0.0097**	39	9.7 (4.3–75)	8.4 (4.4–57)	−1.3	0.067
Cardiovascular Disease (QRISK3)										
Absolute risk, %	159	12.5 (0.1–60.9)	12.7 (0.1–52.6)	0.2	0.17	94	18.3 (1.7–61.0)	19.3 (1.9–53.0)	1.0	0.13
Relative risk	159	2.1 (0.7–11.5)	2.0 (0.8–12.9)	−0.1	**0.0001**	94	2.7 (1.1–11.5)	2.6 (1.1–12.9)	−0.15	**0.0006**

Wilcoxon signed rank test between baseline and latest; bold p value represents statistical significance.
Δ, difference between median at baseline (base) and latest visit; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Fib-4, Fibrosis-4; HDL, high-density lipoprotein; N, number of patients with paired data; NFS, NAFLD fibrosis score; T2DM, type 2 diabetes mellitus.

(FibroScan), a validated surrogate marker of liver fibrosis.[21] Calculation of the Fib-4 score[22] provided additional non-invasive assessment of risk of advanced liver fibrosis. Liver biopsy, still the reference standard for diagnosis and assessment of disease severity, was performed where clinically indicated.

Cardio-metabolic assessment included measurement of weight, body mass index (BMI), alcohol intake, blood pressure, lipids and diabetes control (HbA1c). All patients were asked to complete a qualitative 7-day food diary in advance of clinic attendance. Cardio-metabolic risk factors were defined using standard measures employed in clinical practice: hypertension if blood pressure was ≥140/90 mm Hg, or if the patient was taking antihypertensive medication; type 2 diabetes according to WHO criteria (HbA1c≥48 mmol/mol), if the diagnosis was already established at baseline or if patients were taking anti-diabetic medication; dyslipidaemia according to local thresholds (triglycerides>1.7 mmol/L and/or high-density lipoprotein (HDL) cholesterol <1.0 mmol/L (male),<1.3 mmol/L (female) and obesity if BMI was ≥30 kg/m^2. All patients had 10-year cardiovascular risk assessment (absolute and relative risk) calculated retrospectively using the QRISK3-2017 score[23] and each patient's current drug history was recorded at clinic visits. For patients with T2DM, data on prescribed medical therapies were collated from clinic letters and cross-referenced with written clinic proformas.

The primary outcome was change in ALT level between baseline and latest clinic visit. Secondary outcomes were change in weight, glycated haemoglobin (HbA1c), AST, lipid profile (total cholesterol, HDL, triglycerides), systolic blood pressure, transient elastography, Fib-4 score, NFS and QRISK3 cardiovascular risk score. Patients with a missing variable value at baseline and/or latest clinic visit were not included for paired analysis of that variable. A subgroup analysis was performed on patients with (1) T2DM at baseline and (2) poorly controlled T2DM at baseline, defined as baseline HbA1c>58 mmol/mol, who also had a latest clinic HbA1c measurement.

Economic analysis and assessment of QALE

The UK Prospective Diabetes Study (UKPDS) Outcomes Model (V.2.0, UKPDS-OM2), a lifetime simulation model for patients with T2DM which has been extensively validated, was used to model and predict changes in QALE as well as the potential cost-effectiveness of our approach.[24] UKPDS-OM2 was first applied to all patients with T2DM and subsequently in those patients with poorly controlled T2DM at baseline (HbA1c>58 mmol/mol). The model, incorporates phenotypic data and changes to various modifiable cardio-metabolic risk factors over time. A standard, accepted modelling approach was adopted to compare outcomes in the changes observed in those attending the clinic to

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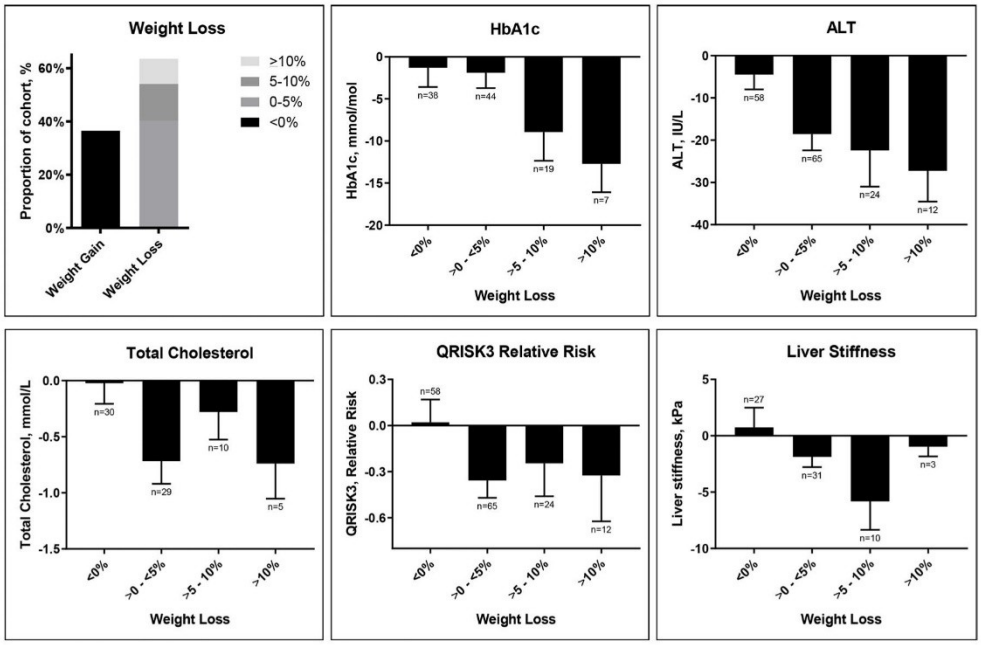

Figure 1 Change in weight and change in cardio-metabolic and liver health stratified by weight loss: baseline to follow-up. ALT, alanine aminotransferase.

outcomes in the absence of these changes (see online supplementary materials for in-depth description).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (V.7.0). Data were non-parametrically distributed as assessed by the D'Agostino-Pearson omnibus test. Continuous variables are quoted as median (range), and categorical variables as numbers and percentages. Non-parametric tests were used to assess for statistical significance (Wilcoxon signed rank test for paired baseline and latest visits, Mann-Whitney U and χ^2 tests for comparing between the T2DM and non-T2DM subgroups) and the threshold for significance was set at the 5%. Endpoints were set as baseline to latest follow-up and response.

RESULTS

Baseline characteristics

165 patients with NAFLD were followed from baseline until their latest clinic visit. Baseline characteristics are described in table 1. Median interval between baseline and latest follow-up clinic visit was 13.3 months (range 2–34), with a median of two follow-up visits (range 1–5).

Patients reported taking a median of four prescription medications (range 0–17) at baseline. Oral hypoglycaemic agents were the most commonly prescribed medication with 52% taking ≥1 agent, followed by anti-hypertensive therapy (51%) and statin therapy (42%).

87 patients (53%) had a liver biopsy prior to or during the follow-up period (table 2). There was no significant relationship between T2DM status and histological NAFLD activity scores (NAS) (p=0.75). Median NAS in patients with T2DM and without T2DM were both five. There was also no significant relationship between T2DM status and fibrosis stage (p=0.19) although the majority of patients with biopsy-proven cirrhosis in the cohort had T2DM (13/16, 81%).

Changes to liver and cardio-metabolic health: baseline to latest clinic visit in all patients

At latest follow-up, median ALT had reduced significantly by 11 IU/L and median AST reduced significantly by 7 IU/L (table 2 and figure 1). Median liver transient elastography also reduced significantly by 1.3 kPa. For Fib-4 and NFS, there were no changes in the proportion of patients switching between categories (risk of presence or absence of advanced liver fibrosis or indeterminate score) (Fib-4: p=0.8, NFS: p=0.7).

Analysis of weight changes revealed that the majority of patients lost weight. The distribution of weight loss is shown in figure 1. Figure 1 also summarises changes in cardio-metabolic and liver parameters stratified by weight loss. In this retrospective analysis of clinical data, even modest weight loss of <5% was associated with improvement in these parameters and suggested that the magnitude of improvement was greater with increased weight loss.

QRISK3 10-year cardiovascular relative risk reduced significantly by 0.1 (5%). There was no significant

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change in QRISK3 10-year absolute risk from baseline to latest visit, though when follow-up age, a component of this algorithm, was corrected to 'age-match' (remain the same) to baseline, this reduced significantly by 0.8 (6%).

Additional analyses of subjects by fibrosis stage (early fibrosis and advanced fibrosis) and by weight response (weight gain or weight loss) were also performed to gain an insight into improvements in liver and metabolic health. These results are summarised in online supplementary tables 1 and 2.

Changes to liver and cardio-metabolic health in patients with T2DM

T2DM is strongly associated with NAFLD progression,[5] and therefore a subgroup analysis of patients with T2DM was performed (tables 1 and 2).

At baseline, patients with T2DM were older than patients without T2DM ($p<0.0001$), and as expected, had higher baseline HbA1c ($p<0.0001$) (table 1). Liver stiffness was also significantly higher at baseline in those with T2DM ($p=0.011$). There was a significant relationship between the presence of T2DM and total number of metabolic syndrome components ($p<0.0001$). Those with T2DM had lower total cholesterol at baseline than those without ($p=0.0011$) (table 1). Statin use at baseline was higher in those with T2DM (65%) compared with those without T2DM (42%).

Median HbA1c reduced significantly by 4 mmol/mol between baseline to last follow-up in those patients with T2DM (table 2). Improvement was most marked in patients who had poorly controlled T2DM at baseline (HbA1c>58 mmol/mol, n=43), reducing by 14 mmol/mol (online supplementary table 3). There were also significant improvements in categories of glycaemic control for those with poorly controlled T2DM subgroup ($p=0.0006$) (figure 2). Weight also decreased in patients with T2DM (table 2).

At baseline, 85 (88%) of patients with T2DM were already prescribed oral glucose lowering therapy, 26% were prescribed insulin therapy and 5% were prescribed GLP-1 therapy. 21% of patients with T2DM started GLP-1 therapy over the duration of the study and 20% of patients who were on insulin therapy at baseline had this discontinued. In those who had insulin therapy discontinued (n=5), median HbA1c reduced by 13 mmol/mol from this point to latest follow-up. 15% of patients were commenced on statin therapy. Net changes to medications in patients with T2DM are summarised in online supplementary figure 2.

Changes in QALE and economic analysis in patients with T2DM

For all patients with T2DM, the UKPDS-OM2 model estimated no significant difference in mean life expectancy in the intervention group (clinic attendees) compared with the reference group (baseline measures carried forward) (online supplementary table 4). In those with poorly controlled T2DM, the intervention group had a significantly increased mean life expectancy of 24 days (95% CI 2 to 50 days) compared with the reference group. Similarly, mean QALE was significantly increased by 29 days (95% CI 6 to 55 days) suggesting that patients with poorly controlled T2DM managed through our multidisciplinary clinic approach had significant improvements in both overall and quality adjusted life expectancy.

UKPDS-OM2 was also used to evaluate cost-effectiveness in patients with poorly controlled T2DM. Lifetime (total) costs were £30.0 k in the clinic group and £29.5 k in the reference group who did not attend the clinic, a mean difference of £0.5 k. Lifetime quality-adjusted life years (QALYs) were 11 years in the clinic group and 10.9 years in the reference group, a difference of 0.1 years (29 days). The resulting incremental cost-effectiveness ratio (cost per QALY was £6.1 k (95% CI £0.3 k to £59.3 k) with 91% of model bootstraps runs falling below a cost per QALY threshold of £20 000.

DISCUSSION

Recent UK and European guidelines,[20 25] advise that management of NAFLD should be multifaceted and patients should be managed using a multidisciplinary approach.[9 10] Nevertheless, there is still a paucity of best practice data describing how such services should

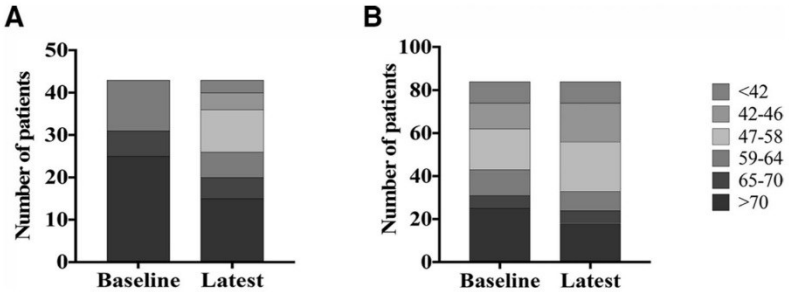

Figure 2 Change in HbA1c in patients with type 2 diabetes mellitus (T2DM): baseline to follow-up. (A) Patients with poorly controlled T2DM (HbA1c>58 mmol/mol) at baseline. (B) All patients with T2DM at baseline.

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be shaped and delivered as well as objective evaluations of the impacts of a multidisciplinary approach on patient outcomes.

Here, we have evaluated our experience of managing a cohort of patients with NAFLD using a dedicated multidisciplinary metabolic hepatology clinic. We describe significant improvements in both clinical and surrogate markers of liver and cardio-metabolic health over a 13-month median follow-up period. This is an observational study of real-life clinical data making it applicable to clinical practice. There was, however, no control arm and the interventions are not uniform for the cohort given the personalised nature of the clinical approach, so causality cannot be inferred. Moreover, such data may be confounded by selection bias and regression to the mean. Nevertheless, it forms a useful benchmark of current clinical practice to which other services or interventions may be compared.

Improvements in markers of liver health

We observed a 14% reduction in liver stiffness in patients attending the metabolic hepatology clinic, measured at point of care and an associated 21% improvement in serum ALT, an insensitive marker of NASH, though one which is still commonly used by healthcare professionals.[26 27] This improvement in ALT is similar to that observed in patients with NAFLD managed through a similar multidisciplinary approach[9] and in lifestyle intervention trials.[28] ALT has also been highlighted as a non-invasive surrogate marker of response in clinical trials in NAFLD/NASH.[29] No other current non-invasive biomarkers, serological or non-serological, have been validated to monitor changes in liver disease severity or to predict liver-related health outcomes, mainly due to poor sensitivity to detect small changes in liver fibrosis.[5] In this cohort, there was no significant improvement in the Fib-4 score. There were also no significant changes in the numbers of patients predicted to have advanced liver fibrosis between baseline and follow-up using non-invasive scores, though the relevance or utility of these findings is not known.

Weight loss and glycaemic control

Both UK NICE[30] and American Diabetes Association (ADA)[31] guidance highlights the importance of 'personalised' or 'individualised' care when managing patients' diabetes taking into account comorbidities and include guidance on weight management. In particular, the more recent ADA guidance outlines that clinicians should consider the weight and cardiovascular risk effects of glucose lowering therapies when escalating diabetes therapy which is particularly important as some newer agents such as GLP-1 agonists and SGLT2 inhibitors have been shown to have beneficial effects on either or both of weight and cardiovascular risk.

In the UK, GLP-1 therapy has a licence for use in patients with T2DM, and NICE guidance[30] outlines that this therapy is recommended in patients with poorly controlled T2DM who are obese, owing to improvements to glycaemic control and promotion of weight loss. Our analysis indicates a reduction in prescriptions for diabetes medications, such as insulin, that are associated with weight gain and an increase in prescriptions for diabetes medications that are weight neutral or associated with weight loss and those which potentially confer cardiovascular protection.

These finding were complemented by overall improvement in glycaemic control for all patients with T2DM, with improvement most marked in those with poorly controlled T2DM. These findings on improvement in glycaemic control in a real-life clinic setting are consistent with previously published studies investigating the impact of diet and or exercise in patients with T2DM. For example, in the Early Activity in Diabetes (Early ACTID) trial in patients with recently diagnosed T2DM who had good glycaemic control at baseline (mean baseline HbA1c 49–50 mmol/mol), HbA1c improved by around 3.3 mmol/mol at 6 and 12 months in those provided with 'dietary support' (dietary consultation every 3 months with monthly nurse support), compared with a control group provided with 'usual care' (initial dietary consultation and follow-up every 6 months—which is often the standard model and frequency of care for patients managed in primary care).[32] Additionally, in this study there was no difference in improvement in HbA1c between the 'dietary support' group and a 'diet plus activity' group (as per diet group, plus 30 min brisk walking five times per week) at either six or 12 months. Previous studies have however shown that a combination of diet and exercise is effective in improving glycaemic control by around 11 mmol/mol at 6 and 12 months.[33] Similarly, in another study, patients with T2DM either provided with specifically tailored food for 1 year or with monthly individual dietary counselling advice saw an approximate 11 mmol/mol reduction in HbA1c over 12 months while a control group receiving standard diabetes care monthly via appointment with a doctor or nurse saw no change in HbA1c over 12 months.[34 35] Improvement in glycaemic control in the scale observed in our study are accepted as likely to improve both diabetes-related and liver-related complications.

Overall, patients achieved a median 3.4% weight loss, though there was considerable variation in response. 23% of patients (37/159 with both baseline and latest weight measurements recorded) lost at least 5% of their baseline weight by latest visit. Previous studies have demonstrated an association between weight loss and improvements in NAFLD, with a reduction in excess of 3%–5% thought to be required for improvement of hepatic steatosis and/or NASH.[36 37] Stratification of markers of liver and cardio-metabolic health by weight response demonstrated that improvements to these markers appear to correlate with degree of weight loss. Our data also suggests that even modest weight loss is associated with improvements in markers of liver

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and cardio-metabolic health. Given the relatively short duration of follow-up, the dynamics of weight loss and any subsequent weight gain were not analysed in this study but would comprise an important follow-up analysis.

Patients often struggle to lose and maintain weight loss without structured support, but there is evidence that commercial weight loss programmes have significant benefit[38] with weight loss of >5% reported, which is of a magnitude that will confer benefit to patients with NAFLD. Liraglutide at a higher dose of 3 mg daily is also licenced for use in patients with obesity without diabetes, but its use in this context is not yet supported through NICE guidance within the NHS.

Finally, our simulation of the lifetime costs and outcomes of the clinic intervention in patients with poorly controlled T2DM indicated that it had a high probability of being cost-effective at the thresholds used by NICE, with an incremental cost per QALY of £6.1 k. While there was no dedicated control group available, a standardised modelling approach was adopted. As such, these findings provide useful additional information and have importance due to the high and growing burden of NAFLD and the need for health authorities to have both clinically effective and cost-effective strategies at their disposal when working within confined financial envelopes.

Significant of this study

What is already known about this subject?

- Non-alcoholic fatty liver disease (NAFLD) is a globally prevalent public health burden with the principal causes of morbidity and mortality being from cardiovascular complications as well as liver disease and cancer. A multidisciplinary approach to the management of patients with NAFLD is advocated but there is a paucity of best practice data describing how such services should be shaped and delivered.

What are the new findings?

- We have evaluated our experience of managing a cohort of patients with NAFLD using a dedicated multidisciplinary approach that is jointly led by hepatologists and diabetologists in a teaching hospital setting with significant input from allied health professionals including diet and lifestyle experts. We describe significant improvements in both clinical and surrogate markers of liver and cardio-metabolic health over a 13-month median follow-up period. These include improvements in liver chemistry and hepatic elastography as well as improvements in weight and glycaemic (diabetes) control.

How might it impact on clinical practice in the foreseeable future?

- This study forms a useful benchmark of current clinical practice to which other services or interventions may in future be compared. In addition, it may help healthcare providers and health policy makers to refine or design and implement healthcare services related to NAFLD, diabetes and metabolic syndrome.

CONCLUSIONS

While considerable effort is going into the development of novel therapies that target the hepatic manifestations of NAFLD/NASH, data from this study indicate that significant improvements in surrogate markers of liver and cardio-metabolic disease can be achieved through effective implementation of existing risk-stratification and treatment strategies. Furthermore, given that NAFLD is both a liver and a cardio-metabolic disease, we have shown that adopting a multidisciplinary approach to its management—involving not only hepatologists, diabetologists and metabolic physicians, but also allied health professionals including diet and lifestyle experts—is a clinically effective and potentially cost-effective way to manage these complex patients.

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