Long-Term Outcomes of Non-Alcoholic Fatty Liver Disease

Thesis submitted in partial fulfilment of the requirement for the degree of Medical Doctorate (M.D.)

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Abstract

Background

Non-alcoholic fatty liver disease (NAFLD) has become a leading cause of chronic liver disease worldwide and affects a third of Western populations. The rising prevalence of NAFLD has been associated with an in increased incidence of complications of cirrhosis such as hepatocellular carcinoma and death and is associated with worrying healthcare and economic burden. The natural history of NAFLD is varied and remains incompletely understood. Long-term, large cohort studies with diversity of disease severity are required to further understanding and improve management of NAFLD.

Aims and Methods

This study aimed to describe the clinical characteristics of a large UK based NAFLD cohort and explore the frequency and predictors of significant clinical events. Participants were identified from the Newcastle Hospitals historical clinical database and the European NAFLD Registry who met the eligibility criteria and had at least 12 months follow up.

Results

Six hundred and five patients were included with a mean follow-up time of 11.8 ± 7.3 years. One hundred and sixteen (19.2%) were cirrhotic at baseline, which increased to 166 (32.9%) by the final clinical event. Co-morbidities such as T2DM, HTN and the metabolic syndrome were common, and the incidence of these rose over the follow-up period. One hundred and twelve patients died over the course of the study; liver disease was the most common cause of death (28.6%). Factors that were prognostic for all-cause mortality included fibrosis stage at baseline (aHR 8.31, 95% CI 4.31-16.01), T2DM (aHR 1.98, 95% CI 1.25-3.14), IHD (aHR 2.31, 95% CI 1.27-4.20) and “high risk” FIB-4 (aHR 10.02, 95% CI 6.14-16.35).

Conclusion

This thesis describes a large, well-characterised NAFLD cohort over a follow-up period of up to 35 years. Factors that predicted adverse outcomes were identified including T2DM, IHD and FIB-4 scores, which could help clinicians identify individuals at risk of poor outcomes.

Acknowledgments and dedication

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Table of Abbreviations

Abbreviation	***Meaning***
AAR	AST/ALT Ratio
AASLD	American Association for the Study of Liver Diseases
ACEi	Angiotensin converting enzyme inhibitor
ADAPT	Age, diabetes, Pro-C3 and platelets
AF	Atrial fibrillation
AIH	Autoimmune hepatitis
aHR	adjusted hazard ratio
ALT	Alanine aminotransaminase
AMA	Anti-mitochondrial antibody
ANA	Antinuclear antibody
APRI	AST to platelet ratio index
ARFI	Acoustic radiation force impulse
ASMA	Anti-smooth muscle antibody
AST	Aspartate aminotransferase
AST/ALT Ratio	Aspartate aminotransferase/Alanine aminotransaminase ratio
AUROC	Area under the receiver operator curve
AUSS	Abdominal ultrasound scan
BAAT	BMI, age, ALT and triglyceride
BARD	BMI, AAR, Diabetes
BMI	Body Mass Index
BP	Blood pressure
BSG	British Society of Gastroenterology
CABG	Coronary artery bypass graft
CAG	Confidentiality advisory group
CAP	Controlled attenuation parameter
CCF	Congestive cardiac failure
95% CI	95% confidence interval
CK-18-M30	Cytokeratin 18- M30
CK-18-M65	Cytokeratin 18-M65

CKD	Chronic kidney disease
CRP	C-reactive protein
CT scan	Computerised tomography scan
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
DPP-4 inhibitors	Dipeptidyl peptidase 4
DSP	Diastolic blood pressure
EASL	European Association for the Study of Liver Disease
ECM	Extracellular matrix
ELF	Enhanced liver fibrosis
ELTR	European Liver Transplant Registry
F (0-4)	Fibrosis stage (0-4)
FDA	Food and drug administration
FIB-4	Fibrosis-4 score
FXR	Farnesoid X receptor
(Upper) GI	Gastrointestinal
GCKR	Glucokinase regulator
GDPR	General data protection regulation
GGT	Gamma glutamyltransferase
GLP-1	Glucagon-like peptide 1
HA	Hyaluronic acid
HAIR	Hypertension, raised ALT, Insulin Resistance index
Hb	Haemoglobin
HbA1c	Haemoglobin A1c
HBV	Hepatitis B virus
HBV sAg	Hepatitis B virus surface antigen
HBV cAb	Hepatitis B virus core antibody
HCC	Hepatocellular carcinoma
HCRW	Health and Care Research Wales
HCV	Hepatitis C virus
HDL	High density lipoprotein
HRA	Health regulatory authority

HSD17B13	Hydroxysteroid 17-beta dehydrogenase 13
HTN	Hypertension
HVPG	Hepatic venous pressure gradient
IHD	Ischaemic heart disease
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Inter-quartile range
LBx	Liver biopsy
LDL	Low density lipoprotein
LSM	Liver stiffness measurement
M probe	Medium probe
MAFLD	Metabolic dysfunction associated fatty liver disease
MBOAT7	Membrane bound O-aceyltransferase 7
MELD score	Model for end-stage liver disease
MetS	Metabolic syndrome
MRE	Magnetic resonance elastography
MRI	Magnetic resonance imaging
MRI-PDFF	Magnetic resonance imaging- proton density fat fraction
MRS	Magnetic resonance spectroscopy
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD Activity Score
NASH	Non-alcoholic steatohepatitis
NASH CRN	National Institute of Health's NASH Committee
NICE	National Institute for Health and Clinical Excellence
NFS	NAFLD fibrosis score
OELF	Original European Liver Fibrosis panel
OR	Odds ratio
OSA	Obstructive sleep apnoea
PCOS	Polycystic ovary syndrome
PIIINP	Procollagen III N-terminal peptide

PNPLA3	Patatin-like phospholipase domain-containing protein 3
PPAR agonist	Peroxisome proliferator-activated receptor agonist
Pro-C3	N-terminal type III collagen propeptide
PVD	Peripheral vascular disease
REC	Research and Ethics Committee
SAF	Steatosis, activity and fibrosis score
SBP	Systolic blood pressure
SD	Standard deviation
SGLT-2	Sodium-glucose co-transporter 2
SSI	Supersonic shearwave imaging
TE	Transient elastography
™	Trade marked
TIMP1	Tissue inhibitor of metalloproteinase-1
TM6SF2	Transmembrane 6 superfamily member 2
TSH	Thyroid stimulating hormone
T2DM	Type II diabetes mellitus
UNOS	United Network for Organ Sharing
U.K.	United Kingdom
US	Ultrasound
USA	United States of America
XL probe	Extra-large probe

Chapter 1: Non-Alcoholic Fatty Liver Disease

1.1 Introduction to NAFLD

1.1.1 Definition

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease characterised by fatty infiltration of the liver. It is classically defined as steatosis affecting greater than 5% of hepatocytes, in the absence of an alternative cause for fat deposition such as chronic viral hepatitis, significant alcohol consumption (frequently defined as >20g/day for women and >30g/day for men), or the use of steatogenic drugs including Amiodarone or Tamoxifen. [1-3]

The term NAFLD is an umbrella used to describe a spectrum of histopathological disease ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) and all stages of fibrosis including cirrhosis.

In 2020 a panel of experts from across the globe proposed a new nomenclature for the condition- metabolic dysfunction associated fatty liver disease (MAFLD).[4] This new term was designed to highlight the close links between metabolic and cardiovascular risk factors and the development and progression of liver disease. However, this name has not yet been adopted by the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of Liver Disease (EASL), the British Society of Gastroenterology (BSG) or National Institute for Health and Clinical Excellence (NICE). The use of MAFLD remains contentious and an international panel has been convened by EASL/AASLD to fully review NAFLD nomenclature. Therefore, for the purposes of this work it will continue to be referred to as NAFLD.

1.1.2 NAFL vs NASH

Non-alcoholic fatty liver (NAFL), previously also known as “bland steatosis” or “simple steatosis”, is a histological diagnosis referring to the presence of fatty infiltration of hepatocytes alone, without any cellular damage.

This differs from non-alcoholic steatohepatitis (NASH) which requires the presence of inflammation and hepatocyte ballooning along with steatosis and is a histological diagnosis.[2, 3, 5, 6]

1.2 Pathogenesis

The pathogenesis of NAFLD is a complex and multifactorial process. For the purposes of this work a simplified overview of the mechanisms believed to contribute to the development and progression of NAFLD will be explored.

Historically a “two hits hypothesis” was understood to be the driver of fatty liver disease. Firstly, there was an increased fat accumulation in hepatocytes due to a sedentary lifestyle and over consumption of calories leading to obesity and associated insulin resistance. This first step predisposed the liver to injury and was then followed by a second “hit” such as oxidative stress, caused by a free radicals produced during fatty acid oxidation for example, which would lead to steatohepatitis and progression to fibrosis.[7, 8]

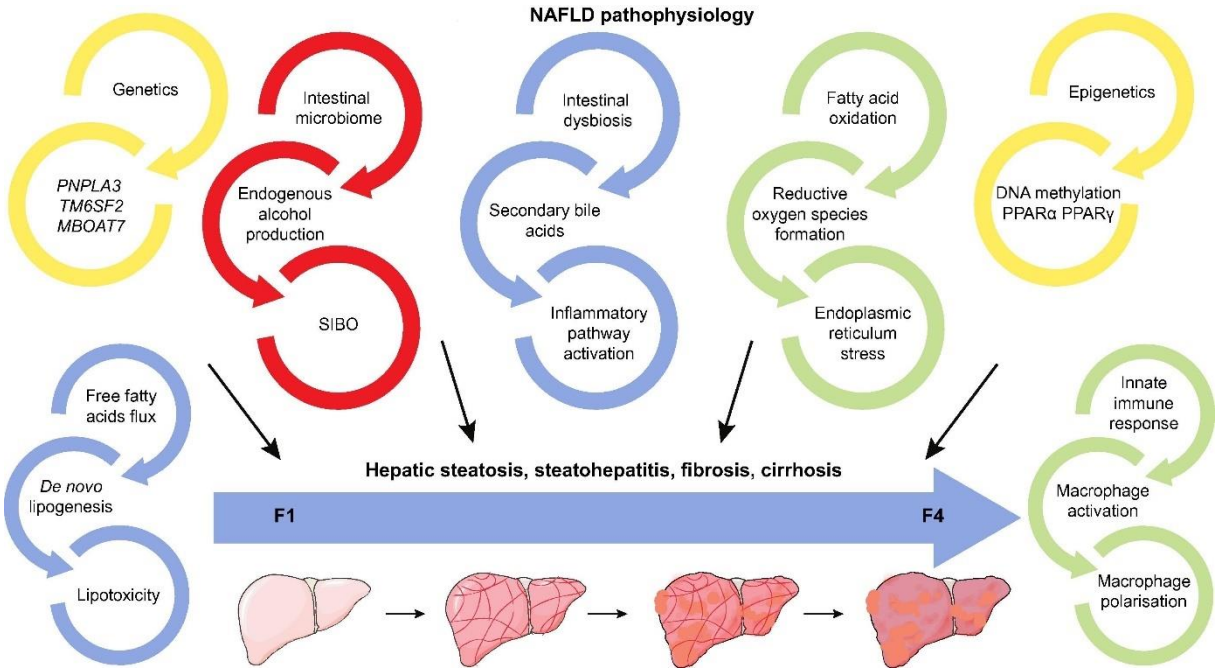
This theory is now out of vogue as it was felt too simplistic for the multiple, simultaneously interacting factors which lead to the development of NASH and has thus been replaced by the “multiple-hit hypothesis”.[7, 8]

This theory again begins with the accumulation of hepatic steatosis as a result of lifestyle, but it now also recognises that there are environmental and genetic components which influence the development of obesity, insulin resistance and hepatic fat.[7-9]

Inflammation and cell death result from the accumulation of lipotoxic lipids as a product of increased free fatty acids from insulin resistance, obesity and altered gut microbiome, leading to a proinflammatory state with hepatocellular stresses (oxidative stress, endoplasmic reticulum stress) and cell death resulting in the cycle of inflammation, tissue regeneration and fibrogenesis.[7-10]

Figure 1.1 below demonstrates some of the factors which interplay in the development of NASH and disease progression in fatty liver disease.

Figure 1.1. Summary of factors which interplay in the development of NASH and are associated with disease progression in NAFLD.



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1.3 NAFLD Aetiology and Associations

NAFLD is strongly associated with other medical conditions such as obesity and insulin resistance and is now recognised as the liver expression of the metabolic syndrome, or a precursor for its development.[12-14]

1.3.1 Obesity

Over the last three decades there has been a large rise in rates of people that are overweight or obese, generally defined as a body mass index (BMI) ≥30kg/m2 in Caucasian populations or ≥25kg/m2 in Asian-Pacific populations, worldwide. This is reflective of an overall increase in weight across general populations, a larger proportion now also fall into the "overweight" category (25-30 kg/m2).[15, 16] The presence of obesity is closely associated with co-morbidities such as type II diabetes (T2DM), the metabolic syndrome and it has been well documented as an independent major risk factor for NAFLD.[2, 3] It is therefore unsurprising that the rising prevalence of NAFLD has been linked to the rising rates of obesity worldwide.[17] A general population study undertaken in two towns in Northern Italy screened the "healthy" population of over 3000 residents for the presence of steatosis. This found an increased prevalence of fatty liver in those who were overweight or obese

compared to those with a normal BMI; 67% of subjects with BMI 25-29kg/m^2, 94% with BMI > 30kg/m^2 compared to just 25% with BMI <25kg/m^2, [18] while others have estimated the prevalence as closer to 80% in the obese population.[19]

While there have not been many studies specifically designed to examine the relationship between obesity and NAFLD, a recent meta-analysis of 21 studies with over 350,000 participants has shown that obese individuals have a 3.5-fold increased risk of NAFLD compared to those of a healthy weight. This has also demonstrated what they describe as a "dose dependent" relationship between NAFLD and BMI.[17] Another meta-analysis concurred with these findings and demonstrated the presence of obesity was associated with a "modest increase" of severe liver disease outcomes (aHR 1.20, 95% CI 1.12-1.28, p<0.001).[20]

However, it is also recognised that NAFLD can occur in patients who are not obese. One recent meta-analysis demonstrated up to 20% of patients can have a healthy BMI.[21] Often referred to as "lean" NAFLD, these patients usually have central adiposity and other risk factors for NAFLD.[22]

1.3.2 Diabetes

Perhaps even more important than the relationship between BMI and NAFLD is the relationship with insulin resistance and Type II Diabetes Mellitus (T2DM). In recent years the complexity of this association is better understood as "bidirectional" with similar underlying pathological mechanisms centred around insulin resistance.[12, 23-26]

Several studies have demonstrated that the prevalence of NAFLD in those with pre-existing T2DM is between 40-70%, with a recent meta-analysis demonstrating an overall global prevalence of 56%. [27-30]

Individuals with NAFLD have also been shown to be at higher risk of developing T2DM than the general population. Several studies have shown a significant increase in the prevalence of T2DM at follow-up compared to baseline.[12, 31] In a small cohort study of 32 people with a mean follow-up of 16.5 years, Nasr *et al*[32] demonstrated the rate of diabetes increased from 6% of the population at baseline to 75% at last follow-up, while McPherson *et al*[33] showed an increase from 48% to 65% in a larger cohort with a median follow-up of only 6.6 years, which is probably a more reflective of a U.K. population.

A recent meta-analysis of 19 observational studies with almost 300,000 participants confirmed this relationship and demonstrated that those with NAFLD have a 2-fold increased risk of T2DM occurrence.[34]

The relationship between T2DM and NAFLD has importantly been shown to be related to poorer outcomes. Patients with the combination of NAFLD and T2DM have been shown to be more likely to have NASH than non-diabetic patients and are at increased risk of developing advanced liver disease and hepatocellular carcinoma (HCC), as well as diabetes related adverse outcomes. [12, 20, 35-38]

1.3.3. Hypertension

Hypertension (HTN) is an extremely common condition estimated to affect ~30% of the general, adult population although it has been reported to increase to as much as 50% in those over 60 years old in England.[39] Several studies have shown that up to 50% of patients with HTN also have NAFLD.[40-42] There has been some debate as to whether an increased blood pressure leads to the development and progression of NAFLD[40, 43, 44], for example a meta-analysis by Singh and colleagues[45] has found the presence of HTN is an independent risk factor for the development of hepatic fibrosis in a cohort of patients. The other side of this debate argues the presence of NAFLD results in an increased incidence of HTN, as suggested in a prospective study of over 20,000 patients which found that the development of HTN was associated with more advanced underlying NAFLD.[46] More recently it has been acknowledged that there is most likely that there may be a bi-directional relationship between the two,[20, 47, 48] although the pathogenesis is not yet fully understood.

1.3.4. Dyslipidaemia

The term dyslipidaemia describes abnormalities in lipid levels which can include *low* high-density lipoprotein (HDL), *high* low-density lipoprotein (LDL), *high* triglycerides, hypercholesterolaemia or a combination of these.[49] It is commonly seen in patients with NAFLD; a global meta-analysis suggests a prevalence of ~70%.[50] Dyslipidaemia is directly involved in the pathogenesis of NASH through the accumulation of lipotoxic lipids and is strongly associated with obesity and cardiovascular morbidity and mortality.[51-53] Studies have shown that the presence of dyslipidaemia correlates with severity of hepatic steatosis[49] and Kanwal and colleagues[54] found in a large population-based study that the presence of low HDL and/or high triglycerides was associated with a hazard ratio 1.23 (95% CI 1.19-1.28) for the development of cirrhosis.

1.3.5. Metabolic Syndrome

There are many varying definitions of the metabolic syndrome (MetS), but a widely accepted definition is the combination of any three of; central obesity, impaired fasting glucose, hypertriglyceridemia, low HDL and hypertension.[55] Table 1.1 below details the definition provided by a joint statement from a multi-specialty meeting with the intent of "harmonizing" the definition of MetS.

Table 1.1 Defining features of the metabolic syndrome.

Feature	Definition
Central obesity	Accepted waist measurements for this may vary with ethnicity UK accepted for Caucasian population: • >94cm for men • >80cm for women
Raised triglycerides	>150mg/dl (1.7mmol/l) Or lipid lowering treatment
Reduced HDL	<40mg/dL (1.0mmol/l) for men <50mg/dL (1.3mmol/l) for women Or on treatment
Impaired fasting glucose	>100mg/dL (5.6mmol/l) Or diabetic medications
Hypertension	SBP >135 mmHg DBP >85mmHg Or use of anti-hypertensive treatment

(55)

As with diabetes, there is also a bidirectional relationship between the metabolic syndrome and NAFLD.[2, 3] Greater than 90% of patients with NAFLD have been shown to have at least one feature of the metabolic syndrome, and 30-40% will have the full syndrome.[56, 57] Younossi and colleagues[50] demonstrated that globally, 42.5% of patients with NAFLD meet the criteria for MetS and this prevalence increases to 70.6% of patients with NASH.

The presence of the MetS is well established as a risk factor for the development of NASH and more advanced disease, and several studies have shown that there is also an increased

risk of mortality in patients who meet the MetS criteria.[20, 52] Stepanova *et al*[53] found that the presence of MetS in patients with NAFLD was associated with an increased mortality risk (aHR 12.08, 95% CI 1.10 – 132.22) and in a recent larger population study Kanwal and colleagues[54] demonstrated that this risk increased with increasing numbers of risk factors; those with T2DM, obesity, hypertension and dyslipidaemia had a HR of 2.56 (95% CI 2.26-2.92).

1.3.6 Genetics

One of the explanations for the wide clinical spectrum of patients with NAFLD is the role that genetics play in the disease. In recent years several genome wide studies have found a number of different genes that influence the outcome of NAFLD and associated risk estimates by 20-70%.[9, 58-61]

PNPLA3 is the most reported gene affecting NAFLD. The I148M variant has been shown to impact all stages of liver damage associated with NAFLD from an increased predisposition to hepatic fat accumulation, to the development of NASH and fibrosis. The presence of this variant is also linked to the development of HCC and one study in particular found after controlling for other factors including age, gender, diabetes, and BMI that the presence of this variant increased the risk of HCC 12-fold.[62-67]

Other genes that play a role in the development and progression of NAFLD include the E167K variant of the TM6SF2 gene which has been found to promote hepatic fat accumulation and increase risk of NASH, fibrosis and HCC[61, 64, 68], the GCKR gene which plays a role in hepatic glycogen synthesis and glycolysis [52, 69] and the MBOAT7 gene which again predisposes to hepatic fat accumulation and more severe liver damage.[64, 70]

Of interest, variants in the HSD17B13 gene have been shown to be protective against the development of liver inflammation and therefore advanced fibrosis and complications of chronic liver disease.[64, 71]

Genetic testing to identify patients at higher risk of disease progression in NAFLD is not yet readily clinically available. However, given there have been genes identified which may predict progression this may well be a tool used in the future to target surveillance and treatment.

1.3.7. Ethnicity

Population studies have demonstrated that the prevalence and severity of NAFLD is also influenced by different ethnic groups.[2, 3, 72] Hispanic populations have been found to have the highest rates of NAFLD and when compared to other ethnic groups such as African American populations. Hispanics also have higher incidences of NASH and more advanced disease, despite the African Americans having higher rates of obesity.[52]

The reasons for this are incompletely understood, and likely multifactorial including social/environmental influences and genetic traits. For example, the re739408 variant of PNPLA3 is associated with significantly higher liver fat content and this is more common in Hispanics but less so in African American populations.[73, 74] Another study compared Latino and non-Latino Caucasian populations and found a higher proportion of the Latino group had NASH and these patients were younger, more sedentary and had a higher carbohydrate intake in their diet compared to the non-Latino Caucasian group with NASH.[75]

Cultural and socio-economic influences also play a significant role in the development of NAFLD. In the Middle East for example, there has been a growth in urbanisation and an associated rise in the availability of the Western diet.[76] This typically includes large portions of energy rich foods, often higher in saturated fats and processed meats than the diet traditional to the region. The Middle Eastern population have also been shown to have reduced physical activity levels when compared to other countries, likely as a result of many factors including a hotter climate and cultural norms.[77, 78] The combination of the change to a Western diet with a sedentary lifestyle has led to a significant increase in NAFLD prevalence in the region.

In recent years the influence of socioeconomics and deprivation on NAFLD has also been recognised. Poverty affects individuals access to healthy food options, facilities for exercise and in countries such as the U.S. where private healthcare systems in place they often have more limited access to routine medical care. In 2021 a study based in the U.S. found that patients with four or more “socioeconomic determinants” such as education level, employment status, access to a vehicle and access to public vs private healthcare were associated with increased severity of NASH.[79]

1.4 Epidemiology and disease burden

Over the last decade, as a result of factors including rising obesity rates and aging populations, NAFLD has become the leading cause of chronic liver disease worldwide.[80] The global prevalence in one meta-analysis was estimated to be at 25%[50], and with this a sharp rise in liver morbidity and mortality due to NAFLD is being seen.[81]

Given NAFLD is often an asymptomatic condition and a challenge to diagnose in some clinical settings where liver biopsy is not readily available, it is likely that the true rates of prevalence are underestimated. The presence of NASH, which is a histological diagnosis, is also greatly underestimated as a result of diagnostic limitations.

In North America, depending on the methodology of diagnosis, studies have estimated 21-24% of the population have NAFLD.[72] A further 21% of these patients are thought to have NASH, accounting for 1.5-6.45% of the general population.[50] When taking into account the size of this population the magnitude of the NAFLD problem can be better understood; 83.1 million people are thought to have NAFLD, 16.5 million NASH.[82] In the USA, NASH is set to become the leading cause of liver transplantation by 2030.[83]

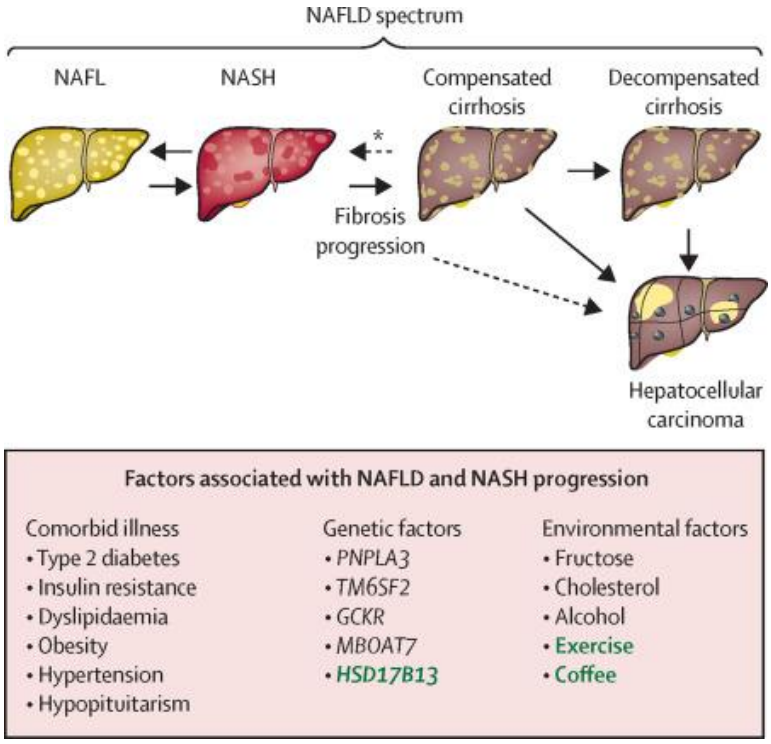
Europe is another area where NAFLD is becoming more prevalent as a direct result of rising rates of obesity, diabetes and the metabolic syndrome.[84-86] The prevalence of NAFLD ranges from 5-44% across the continent and in the UK the current estimate is 21.9-26.4%[50, 82]. One study in a hepatology outpatient department in England reported an incidence of 29 cases per 100,000 person years.[73, 87]

A rise in paediatric rates of obesity worldwide poses another challenge for the future rates of NAFLD. In the USA the prevalence of obesity in children between 2-5 years old rose from 8.4% in 2011-12 to 13.9% in 2015-15[88], and currently 2.6-17.3% of overweight/obese children are thought to have NAFLD globally.[89] Disease onset in childhood is associated with poorer outcomes, the lifetime risk of developing HCC is increased 20-30% in this group.[90]

1.5 Natural history

As described above, the diagnosis NAFLD covers a broad range of disease spectrum with a wide array of disease outcomes. Figure 1.2 below demonstrates the range of stages of NAFLD.

Figure 1.2. Illustration depicting the spectrum of NAFLD and progression from NAFL to NASH, and the development of cirrhosis and its complications.



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It is understood that the majority of patients will have stable NAFL or a slowly progressive disease course which will not result in significant fibrosis or cirrhosis.[9, 80] However, there will be a small proportion of patients who do develop the more aggressive form of NAFLD with liver-related adverse outcomes such as decompensated cirrhosis, HCC or death, and recognising those individuals at risk of progression is a key component of managing the disease. Unfortunately, the natural history is not well understood and is a key area of ongoing research.

Historically it was thought that NAFL was a "benign" form of the disease and not associated with fibrosis progression.[89, 91-93] However, in recent years studies have shown this to be incorrect. Wong *et al*[94] found 28% of patients diagnosed with isolated steatosis had fibrosis progression on repeat liver biopsy only 3 years after the initial histology, Pais and colleagues[95] found 6 of 25 participants with NAFL on index biopsy had developed bridging fibrosis by the time of repeat histology with a mean follow-up of 3.7 years, and McPherson et al[33] found progression to NASH in 44% of patients with baseline NAFL, and 22% of those with isolated steatosis at baseline had progressed to stage 3 fibrosis by the follow-up biopsy 6.6 years later. A meta-analysis exploring fibrosis progression in patients with NAFL vs NASH

agreed with these findings and showed that progression is slower overall in those with NAFL compared to NASH (0.07 stages per year compared to 0.14 stages per year)[45] and factors such as mild inflammation on biopsy in conjunction with worsening metabolic risk factors with time are associated with this disease progression.[95, 96]

The risk of progression of fibrosis in patients is complex with significant heterogeneity seen, making it hard to predict an individual patient's risk of progression.[96] The presence of histological markers of disease activity (inflammation or ballooning) have not conclusively been shown to correlate to disease progression,[45] although Pais *et al* did note baseline inflammation as a potential cause for progression in their isolated steatosis group.[95] The presence, or development of T2DM has been shown to be the single most important factor associated with progression[32] however, other factors including age, HTN, obesity and genetics have all been shown to play a role in the progression of fibrosis.[1, 3, 9, 45]

Long-term follow-up studies in patients with biopsy proven NAFLD in recent years have consistently found the only factor which reliably predicts the development of liver related complications and liver mortality is the presence of baseline fibrosis.[80, 97-100] In a meta-analysis Taylor *et al* found the presence of F3-4 conferred a 5-12 fold increase in relative risk of death or liver related events, including HCC or liver transplantation.[101]

Despite the slow rate of disease progression in NAFLD compared to other aetiologies of liver disease, 15-30% of patients with NAFLD go on to develop NASH cirrhosis.[96] Progression from compensated to decompensated cirrhosis occurs at an estimated rate of 3-4% per year.[9, 102] Ekstedt *et al*[97] demonstrated 5.4% of their cohort developed liver related complications including ascites and hepatic encephalopathy after a mean of 13.7 ± 1.3 years, and Angulo *et al*[99] found 7% of their subjects had a liver related event over a similar mean follow-up, and of particular interest they reported 38.6% of these patients died from these liver complications. Whilst proportionally these numbers seem small it is important to note that these studies had a relatively short follow-up duration. With increasing prevalence of NAFLD, particularly with a rise of paediatric cases, it is likely that the prevalence of decompensated cirrhosis will continue to rise as a result of prolonged risk exposure and aging populations.[96] In the USA, decompensated NASH cirrhosis as an indication for liver transplant has increased 170% over the last decade.[83]

Hepatocellular carcinoma (HCC) is another key liver related complication associated with NAFLD.[103] Whilst the incidence of HCC in patients with NAFLD is reported as lower than those with liver diseases such as viral hepatitis or alcohol related liver disease, with the high global prevalence of the disease, NASH related HCC has become the most common cause of HCC in some areas, such as the North East of England.[37, 96, 104] The incidence of HCC in patients with NAFLD is reported to range between 2.4% to 12.8%, and importantly it is also recognised that 20-30% of NAFLD HCC cases arise in the absence of cirrhosis.[9, 105, 106] The pathogenesis of this is not fully understood but thought to be linked to the increased association with obesity and malignancy. Patients with NAFLD-HCC are generally older compared to those with other aetiologies and as other co-morbidities, such as cardiovascular disease, are frequently present, they have poorer outcomes and are more likely to die as a result of the HCC diagnosis.[37, 80]

1.6. Diagnosis of NAFLD

1.6.1 Clinical index of suspicion

The majority of patients with NAFLD are asymptomatic until they develop advanced liver disease, and therefore clinicians must have a high index of suspicion in those who present with other features of the metabolic syndrome, in particular obesity and diabetes. The European Association for the Study of the Liver recommend screening patients with MetS for NAFLD as >80% of patients with central obesity have been found to have hepatic steatosis, and this is as high as 70-90% in those with diabetes.[1-3, 107]

In order to make a diagnosis of NAFLD, a detailed clinical history must be taken to exclude other causes of steatosis. A sole diagnosis of NAFLD cannot be made in conjunction with another underlying cause of chronic liver disease such as viral hepatitis or auto-immune liver disease, and the use of steatogenic drugs such as Tamoxifen or Amiodarone must be excluded. Finally, a detailed alcohol history must be taken to exclude potentially harmful alcohol consumption as the cause of steatosis. Individuals must not regularly consume alcohol exceeding 20g/day for women and 30g/day for men to be diagnosed with NAFLD.[1-3, 107]

1.6.2 Blood Tests.

As most patients with NAFLD are asymptomatic, the majority are uncovered incidentally after a discovery of raised liver bloods.[56, 107] The most common liver blood abnormality seen in patients with NAFLD is raised gamma glutamyltransferase (GGT) levels, however this is not exclusive to this condition and can also be elevated in excess alcohol consumption and with the use of some drugs.[108, 109]

Raised transaminases (ALT > AST) are also commonly elevated in patients with NAFLD, however this is not reliable as up to 80% of patients can have normal ALT levels (<35-50IU/L depending on laboratory ranges).[110] This may be due in part to the known association of falling ALT levels with disease progression and increasing age.[111] Prati *et al* [112] proposed the use of revised laboratory limits (<30IU/l for men and <19IU/l for women), which may improve diagnostic sensitivity but not specificity. AST has not been shown to have any significant relationship with age, but levels are known to increase with advancing stage of liver disease, although again this is not specific to NAFLD, and elevated levels of AST can also be found in alcohol related liver disease or autoimmune hepatitis (AIH) as well as heart disease and smooth muscle disorders.[108]

Elevated liver blood tests in a patient with risk factors for NAFLD, such as obesity and diabetes, suggest a diagnosis of fatty liver but a full blood liver screen should also be undertaken to rule out any other causes of liver disease or coexisting conditions.

Other blood tests in the liver screen which may be elevated in patients with NAFLD include serum immunoglobulin A (IgA) which is seen in 46% of patients with NAFLD and has also been found to be an independent predictor of advanced disease.[113]

Low levels of autoantibody positivity can also be seen and in particular antinuclear antibody (ANA) >1:160 and or anti-smooth muscle antibody (ASMA) >1:40 have been reported in 21% of cases in a study of over 850 patients with histologically confirmed NAFLD.[114] The presence of autoantibodies has not been shown to be associated with more advanced disease but may prompt the need for a liver biopsy to confirm the diagnosis and exclude the presence of an alternative or concurrent autoimmune liver disease diagnosis.

Finally high ferritin levels are commonly seen in patients with NAFLD. The elevated ferritin levels in these patients are due to underlying inflammation or insulin resistance rather than

iron overload and can be indicative of more advanced fibrosis.[115, 116] Transferrin saturations should be assessed to exclude Haemachromatosis in individuals with a raised serum ferritin.

1.6.3 Imaging

The other common pathway whereby NAFLD may be discovered is by the incidental finding of hepatic steatosis on radiological imaging such as an abdominal ultrasound scan (AUSS).

AUSS is the most commonly used first line imaging investigation to look for steatosis as it is widely available, low cost and provides no radiation risk to patients.[56, 107] A "fatty-liver" will appear bright on US due to increased hepatic echogenicity.[117] However, ultrasound is an operator dependent investigation, and it is only accurate at detecting steatosis affecting greater than 20-30% of hepatocytes.[118, 119] It can be insensitive for mild steatosis and also miss steatosis in patients with cirrhosis where the hepatic fat levels are known to reduce or "burn-out".[56] It can also be difficult to differentiate steatosis from fibrosis on ultrasound. This therefore means the absence of hepatic steatosis on an ultrasound does not exclude the diagnosis of NAFLD and in patients with a high degree of clinical suspicion further imaging or investigations are recommended.[119]

Controlled attenuation parameter (CAP) is another ultrasound-based technology which is performed alongside transient elastography by Fibroscan™ and has been found to be more sensitive in detecting the presence of hepatic fat. This technique involves the use of a shear wave propagated through the liver which is altered in the presence of steatosis. In one of the first studies exploring the use of CAP in 115 patients of mixed aetiology it was shown to accurately detect >10% steatosis (AUROC 0.91).[119, 120] One of the early limitations of this technique was the failure of CAP in some patients with a high BMI,[121] however, failure rates have been reduced with the introduction of the XL-probe. A recent meta-analysis assessing use of CAP for hepatic steatosis assessment concluded that the optimal cut-offs have not been determined and CAP does not have the diagnostic precision to differentiate between specific grades of steatosis (S0-3) but it may have some value in screening for NAFLD given its ability to detect steatosis.[122] The final major limitation for this technique is the clinical availability of Fibroscan™ which is currently still largely only accessible in secondary care settings in the UK.

Steatosis can be detected on CT scan, and this is another common incidental means by which the presence of hepatic steatosis is often discovered. However, as with ultrasound,

this is most accurate when there is >30% steatosis and given the clinical cost, availability, and associated radiation with this imaging methodology it is not commonly used to investigate a potential NAFLD diagnosis.[123, 124]

Magnetic resonance imaging (MRI) is the most accurate non-invasive modality for the diagnosis of hepatic steatosis.[125, 126] There are two main methods utilised by MRI to identify the presence of fat accumulation in the liver: MR spectroscopy (MRS) and MRI-proton density fat fraction (MRI-PDFF). MRS uses differences in resonance frequencies of protons in water vs fat in an area of the liver to quantify liver fat. It is able to assess a larger area of liver than a biopsy can and has been used in clinical trials to measure liver fat in large populations and to monitor changes in fat with therapeutic interventions.[110, 127-131] However, given the specialist nature of the imaging it is not utilised widely in a clinical setting. MRI-PDFF is a newer technique based on the same assessment of protons bound to fat vs water in the liver, but the major advantage with this method is the ability to assess the liver in its entirety for steatosis, thus overcoming sampling variability.[132] Studies have shown that MRI-PDFF correlates well with histology and can accurately diagnose the presence or absence of steatosis with an area under operator receiving curve (AUROC) 0.989 [119, 133, 134]. There was also better agreement between radiologists reporting the MRI than histologist reporting liver biopsy results.[135] As with MRS, MRI-PDFF is effective in monitoring the response to therapeutic interventions in clinical trials.[129, 136-138] However, given the cost and availability of this technique it is not widely used clinically and is largely still a research tool.

1.7 Staging of disease: NAFL vs NASH

Differentiating between NAFL and NASH is an important step in the assessment of a patient with fatty liver disease as NASH is known to affect 10-30% of patients and is associated with more progressive disease leading to cirrhosis in up to 20%.[28, 56, 92, 94, 139] NASH is a histological diagnosis based upon the presence of steatosis with hepatocellular injury and inflammation. Several studies have attempted to develop novel biomarkers, clinical models, and imaging techniques in order to discriminate NAFL from NASH using non-invasive techniques but unfortunately to date none have been found to be reliable for clinical use yet.[140-142]

The biomarkers and clinical models trialled thus far have endeavoured to focus on various aspects of the pathogenesis of NASH, table 1.2 below summarises some of the blood-based biomarkers and panels that have been developed and their components.

Table 1.2. Summary of biomarkers developed to assist in the diagnosis of NASH, differentiating from NAFL.

Test	Components	Purpose	AUROC	Sens, Spec	References
CK-18 • CK-18-M30 • CK-18-M65	CK-18 is the major filament protein making the cytoskeleton structure of hepatocytes. During apoptosis cleaved fragments of this protein are detectable in the blood stream.	NASH diagnosis	• 0.82 • 0.80	66-83%, 75-98%	(127, 140-143)
NASH Test	Age, sex, BMI, serum triglycerides, cholesterol, alpha2macroglobulin, apolipoprotein A1, haptoglobin, bilirubin, GGT, ALT, AST.	NASH diagnosis	0.79	33%, 94%	(127, 144, 145)
NASH diagnostics	There are several studies describing "NASH diagnostics" with overlapping panel components which include: CK-18, adiponectin, Resistin (an adipose derived hormone), IL6, T2DM, sex, BMI and triglycerides	NASH diagnosis	0.73 0.81 0.90	71.4%, 72.7% 91%, 92%	(127, 142, 145-148)
oxNASH	13 hydroxyl octadecadienoic acid/linoleic acid ratio, age, BMI and AST	Risk for NASH diagnosis	0.83	81%, 97%	(142, 149)
NASH ClinLipMet score	AST, PNPLA3 genotype, fasting insulin, glutamate, isoleucine, glycine, lysophosphatidylycholine 16:0, phosphoethanolamine 40:6	Predict NASH	0.87	85.5%, 72.1%	(142, 150, 151)
OWLiver	28 triglycerides and BMI	Discriminate NAFL vs NASH	0.90	83%, 94%	(151, 152)

1.8 Staging of disease: fibrosis

As fibrosis stage has been shown to be the most important predictor of long-term outcomes in patients with NAFLD, it is vitally important to assess this at the time of diagnosis. This allows for appropriate surveillance of patients and timely intervention to reduce the risk of adverse liver outcomes such as progression to cirrhosis and complications, including HCC. Historically, a liver biopsy was the only means by which fibrosis could be accurately assessed, but there have been various "non-invasive" methods developed with varying degrees of success in accurately staging fibrosis.

1.8.1 Non-invasive "simple" scores

Several simple non-invasive systems have been developed using readily available clinical data such as blood results, anthropometric measurements, and co-morbidity statuses. The primary benefit of such scores is they can be easily calculated in a clinical setting with very little cost or expertise required.[107]

The *AST/ALT Ratio (AAR)* is one of the oldest and simplest tools to stage fibrosis. It is based on the premise that as fibrosis stage progresses to cirrhosis there is an associated fall in ALT levels, whilst the AST levels remain the same or increases.[153] This results in a raised AAR in patients with advanced liver disease, and this was being used as early as 1967 to differentiate between alcoholic liver disease and acute hepatitis.[154, 155] McPherson and colleagues validated the use of the AAR in a NAFLD specific cohort and found using the threshold <0.8 could reliably exclude advanced fibrosis with an AUROC 0.83 and could avoided a liver biopsy in 69% of patients in their cohort.[156] However as discussed above, ALT levels are affected by increasing age and this will have an impact on the accuracy of the AAR in patients >65 years old.[111]

The *AST to Platelet Ratio Index* (APRI) was developed in 2003 with the intention of predicting significant fibrosis or cirrhosis in patients with Hepatitis C. Wai *et al*[157] proposed that the index would magnify the effects of liver fibrosis on AST and platelet levels and in their initial derivation cohort the APRI score was found to predict significant fibrosis and cirrhosis with and AUROC 0.80 and 0.89 respectively. It has been evaluated in the NAFLD cohort by several different studies and several "optimal" cut-offs have been proposed, but overall it has not been found to perform as well in the NAFLD cohort.[127] When the APRI was assessed in the

same cohort from McPherson *et al* it only achieved an AUROC 0.67 with 27% sensitivity and 89% specificity.[156]

The FIB-4 Score was originally developed to predict liver fibrosis in patients with HIV/HCV co-infection.[158] Using features found to be associated with advanced fibrosis they developed the following formula:

$$Age\ (years) \times AST\ (IU/L) / Platelet\ (10^9/L) \times \sqrt{ALT\ (IU/l)}$$

When compared to 6 other non-invasive tools in a NAFLD cohort Shah and colleagues[159] found the FIB-4 score performed the best at diagnosing/excluding advanced fibrosis (F3-4) with an AUROC 0.802. Using the cut-off <1.3 had a 90% negative predictive value for F3-4, and a score >2.67 had an 80% positive predictive value, leaving only a quarter of the cohort in the "indeterminate" range.[159] When McPherson *et al*[156] compared the FIB-4 in their cohort they demonstrated that 62% of patients could have avoided a liver biopsy using the <1.3 cut-off, and there have been several other studies which have demonstrated the FIB-4 score outperform others in the NAFLD cohort.[127, 160]

One limitation to the FIB-4 is the use of ALT in the algorithm given the known impact of age on this test. For this reason, alternative cut-offs for those over 65 years old have been suggested (<2.0 advanced fibrosis excluded, >2.67 advanced fibrosis likely) which has improved the specificity of the results and resulted in fewer patients requiring unnecessary further investigation and this has been endorsed by the British Society of Gastroenterology in their guidelines for NAFLD management.[111, 161]

In contrast to the tools above the *NAFLD Fibrosis Score* was developed with the specific goal of diagnosis or exclusion of advanced fibrosis (F3-4) in the NAFLD population.[162] Using a multicentre study with over 700 biopsy confirmed NAFLD patients, including some from the Newcastle upon Tyne Hospitals, a scoring system was created from routinely available clinical and laboratory results that were found to independently predict the presence or absence of advanced fibrosis. These were age, BMI, the presence (or absence) of diabetes, AAR, platelet count and albumin levels, and the below formula was derived:

$$-1.675 + 0.037 - age + 0.094 - BMI + [1.13 \times diabetes\ (y=1, n=0)] + [0.99 \times AAR] - [0.013 \times platelet] - [0.66 \times albumin]$$

Angulo and colleagues suggested the use of <-1.455 as a low cut-off to reliably exclude advanced fibrosis with a 93% negative predictive value, and >0.676 as a high cut off to

accurately diagnose advanced fibrosis with a positive predictive value of 90%. The NFS has been validated in several other studies[163-165] including by McPherson *et al* who found it performed well in comparison to the other tools with an AUROC 0.81, and its use could have reduced the need for a liver biopsy in 52% of their cohort.[156]

As with the FIB-4 score, the NFS results are adversely affected by age and therefore alternative cut-offs for its use in patients >65years old have also been suggested.[111] Using <0.12 as the lower cut-off improves the specificity of results from 20% to 70% in this age group without impacting on sensitivity and thus this new cut-off is also supported by the BSG guidelines.[161]

There have been many more simple non-invasive panels developed to diagnose or exclude advanced fibrosis including BARD[166], BAAT[167] and more recently ADAPT[168]. These have been shown to perform similarly to those discussed above but are less established and not currently used in the Newcastle upon Tyne Hospitals NAFLD clinic which focuses on the 4 scores listed above as currently recommended by the European and AASLD guidelines.

1.8.2 Panels/biomarkers

A number of biomarker panels have also been developed to identify fibrosis in NAFLD and the most widely validated for clinical use is the ELF panel. Currently the ELF panel is the only FDA approved biomarker (although for prognostic use, not as a diagnostic) and is recommended by NICE guidelines as a method of screening for advanced fibrosis.[169] The panel consists of 3 markers of cell matrix turnover: type III procollagen peptide (PIIINP), hyaluronic acid (HA), and tissue inhibitor of metalloproteinase-1 (TIMP1) and in 2008 Guha and colleagues[170] reported the ELF panel could accurately distinguish advanced fibrosis (F3-4) from F0-2, AUC 0.90 (95% CI 0.84 – 0.96, sensitivity 80%, specificity 90%, NPV 94%), marginally outperforming the NAFLD Fibrosis Score. The ELF score has been assessed in several other studies, including the recent multi-national STELLAR clinical trial which found that the ELF score performed better than FIB-4 or NFS (AUROCs ELF=0.80, FIB-4= 0.78, NFS=0.74).[171] This trial also demonstrated that the ELF score, and its change over time, was associated with disease progression in patients with bridging fibrosis and cirrhosis at the baseline visit (HR 2.11, 95% CI 1.53 – 2.90, p<0.001),[172] which may have very interesting clinical possibilities but requires further exploration before its use as a predictor of outcomes can be implemented. More recently in a systematic review by Vali *et al*[173] the ELF panel was again shown to perform with high sensitivity but limited specificity in excluding advanced

fibrosis. This group identified the performance of ELF was significantly impacted by the population being tested, i.e., the underlying disease prevalence, and therefore alternative cut-offs were proposed in different clinical settings to improve specificity when used in a primary care setting for example. As with its use for prognostication, these alternative cut-offs for different clinical settings will require further validation prior to implementation in a clinical setting.

Whilst biomarker panels such as ELF show significant promise in aiding the staging fibrosis in NAFLD, they are expensive when compared to the simple non-invasive scores above and therefore not routinely available in clinical settings, but often used as a second line tool.[107] Table 1.3 below details some of the more commonly used biomarkers and commercial fibrosis panels.

Table 1.3. Summary of biomarkers and commercial fibrosis panels developed to identify or exclude fibrosis in NAFLD.

Test	Components	Aim	AUROC	Sens, Spec	Reference
ELF panel	Hyaluronic acid (HA), tissue inhibitor of metalloproteinase-1 (TIMP-1), amino-terminal peptide of pro-collagen III (P3NP)	Diagnose the presence of: • Moderate fibrosis (F2-4) • Severe fibrosis (F3-4)	• 0.90 • 0.93	• 70%, 80% • 80%, 90%	(170)
Pro-C3	Pro-C3 is a neo-epitope marker of type III collagen formation used in fibrogenesis	Detection of advanced fibrosis	0.76-0.83	60-72%, 71-74%	(168, 174)
Fibrometer (NALFD)	Age, weight, glucose, AST, ferritin, ALT and platelet count.	Detecting F3-4 (using >0.715 threshold)	0.94	79%, 96%	(175)
Fibrotest	Alpha-2 macroglobulin, apolipoprotein, haptoglobin, GGT, bilirubin, age and sex	Detecting the presence of ≥F2 (using <0.30 threshold)	0.81	77%, 77%	(176)
Hepascore	Age, sex, bilirubin, GGT, HA and alpha2-macroglobulin	Diagnosis of ≥F3 (using	0.81	76%, 84%	(177-179)
SOMAScan	Proteomics-based model comprising of either 4 or 12 proteins.	Differentiate between advanced fibrosis in patients with NASH	• 0.74 • 0.83		(180)
ADAPT	**A**ge, **dia**betes, **P**RO-C3 and pla**t**elet count	Identification of advanced fibrosis	0.86	90.9%, 72.7%	(168)

1.8.3. Imaging

Ultrasound and CT can detect features classically seen in established cirrhosis such as a nodular appearance to the outline of the liver, coarseness of the liver parenchyma, enlargement of the caudate lobe or signs of portal hypertension however, they are unable to detect earlier stages of fibrosis and subtle changes of early cirrhosis and therefore are not used to assess stage of NAFLD.[127, 181]

Over the last two decades several different technologies have been developed to allow accurate non-invasive staging of liver disease, including NAFLD. The most commonly used method in the UK is Transient Elastography (TE). TE (also referred to as Fibroscan™) is an ultrasound-based investigation which uses a pulsed-echo ultrasound to measure the velocity of a shear wave being transmitted through an area of the liver. This generates a "liver stiffness measurement" (LSM) which has been shown to correlate well with histological fibrosis stage in various aetiologies of liver disease.[119, 182, 183] One of the early limitations of Fibroscan™ in the NAFLD cohort was the failure rate due to obesity, and this has been significantly improved with the introduction of the XL probe.[184] This probe uses a lower frequency ultrasound wave which is able to generate a deeper area of interest and improves the accuracy of readings in patients with central obesity.[185, 186] A meta-analysis of >1000 NAFLD patients showed that TE had excellent accuracy in diagnosis cirrhosis (92% sensitivity and specificity), good accuracy for F3 (85% sensitivity and 82% specificity), but only modest accuracy for F2 (79% sensitivity and 75% specificity).[187] There remains debate over the optimum cut-offs, and there are differences in these based upon the use of the M vs XL probe, but in general <8.0kPa (<7.2kPa on XL) reliably excludes advanced fibrosis (F3-4) and >9.6kPa suggests F3-4.[119, 188] TE does have some other limitations including operator variability and the validity of the results can be influenced by post prandial increases in portal blood flow, hepatitis and the presence of ascites. However, Fibroscan™ is a quick, painless, and increasingly clinically available tool and for these reasons it is a good second line investigation to confirm advanced fibrosis.

Acoustic radiation force impulse (ARFI) is another ultrasound based technology used to assess liver fibrosis. ARFI uses "B-mode" ultrasonography to generate an ultrasonic pulse to "excite" liver tissue and result in a shear wave, the velocity of which is measured to give an indication of fibrosis stage: an increase in median velocity is associated with increasing severity of fibrosis.[119, 127, 189] There are a few different ARFI techniques available from

different ultrasound manufacturers and they all perform differently in the assessment of fibrosis. The best studied in NAFLD is the Virtual Touch™ Quantification, which is available on most standard Seimens ultrasound machines.[107] In a systematic review of seven studies including over 700 NAFLD patients, ARFI (Virtual Touch) was shown to have reasonable accuracy in diagnosing clinically significant fibrosis (≥F2, AUROC 0.898).[190] However, as with TE, there remains debate as to the optimum cut-offs and further data is required to improve its clinical usability.[191-193]

Supersonic shearwave imaging (SSI) is another variation of ultrasound elastography which uses an ultrafast high frequency scanner which can be integrated into a normal ultrasound machine.[107, 189] A study of 291 patients with biopsy proven NAFLD showed that SSI performed slightly better than TE and ARFI, but further validation is required.[192]

Magnetic resonance elastography (MRE) is the most accurate non-invasive method of staging fibrosis in NAFLD.[194] MRE uses a modified phase-contrast pulse sequence that allows visualisation of a shearwave into the liver tissue and can assess the whole liver.[119, 195] Loomba *et al*[194] showed that MRE was very accurate in discriminating between F3-4 and F0-2 (AUROC 0.924), and this was supported by a systematic review of nine studies that found AUROCs for each fibrosis stage were ≥F1 = 0.86, ≥F2 = 0.87, ≥F3 = 0.90 and F4 = 0.91.[196] This technique is also being evaluated in clinical trials to monitor response to treatment.[107, 197] As with other MRI tools its use is largely in research settings.

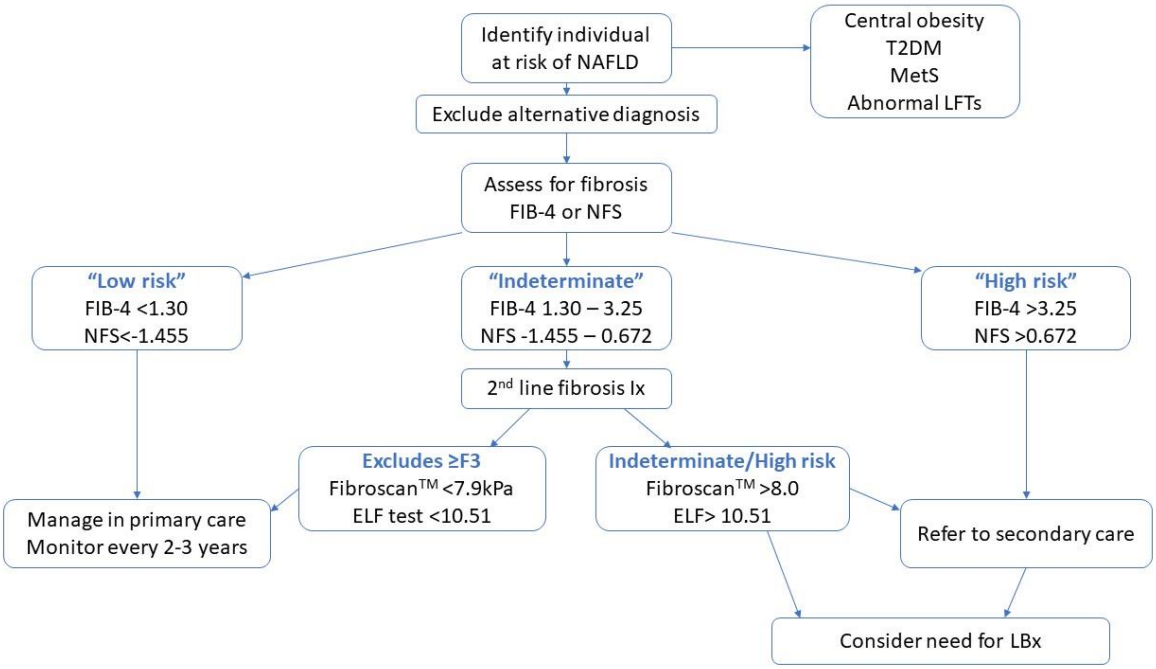
There have been two studies that have compared the performance of TE to MRE for the staging of NAFLD fibrosis. In both studies MRE was found to be superior: Imajo *et al*[198] found MRE outperformed TE in the diagnosis of F2 (AUROC 0.91 vs 0.82) and F4 (AUROC 0.97 vs 0.92), while Park and colleagues[199] found MRE performed better across all stages of fibrosis (F1-4) with an AUROC 0.82 compared to 0.67 for TE. In particular these studies highlighted the improved performance of diagnosis of earlier stages of fibrosis with MRE than TE.

1.9 Histology

Despite the advances in the above investigations to detect steatosis, exclude other causes of liver disease and detect and stage steatohepatitis and fibrosis, the “gold-standard” investigation for the diagnosis and staging of NAFLD is liver biopsy.[1-3]

In the U.K., current clinical practice would seek to use non-invasive tests such as the FIB-4 score as a first line method to assess for advanced fibrosis. At this stage, those who fall into the "low risk" category can safely be managed in primary care and monitored every 2-3 years for disease progression with repeat non-invasive testing such as the FIB-4 score, while those deemed "high risk" should be referred to secondary care. Individuals who fall into the "indeterminate" category should undergo a second line non-invasive test, such as a Fibroscan™ or ELF panel depending on availability and clinician preference, to further assess fibrosis stage. If this second line investigation again does not conclusively exclude advanced fibrosis or there is concern as to the underlying diagnosis then referral to secondary care should be made and a liver biopsy should be considered to clarify and stage the liver disease.[161, 200, 201] Figure 1.3 below demonstrates this pathway.

Figure 1.3. Flow diagram demonstrating the current BSG guidance on a practical, clinical approach to staging NAFLD by use of non-invasive tools such as the FIB-4 score or Fibroscan™ as a first line tool.



1.9.1 History of NAFLD histology

NAFLD is generally acknowledged to have been recognised clinically as a form of chronic liver disease independent from alcohol related liver disease by Ludwig *et al*[202] in 1980 and the histological features were described by various different studies over the following years.[203, 204] The Brunt Criteria[205] proposed in 1999 was the first widely used system to grade and stage the histological features of NASH. This criteria was developed based on the most

significant histological features found on a review of liver biopsies from 51 patients already diagnosed with NASH.

In 2005 Kleiner and pathologist colleagues in the National Institute of Health's NASH Committee (NASH CRN) further developed the original criteria and proposed a grading system called the NAFLD Activity Score (NAS)[206]. This was developed using 50 biopsies and 9 pathologists to review the variability between reporting and the features with the greatest reproducibility (steatosis severity, hepatocellular ballooning and lobular inflammation) were chosen to create the NAS score, with the intention of monitoring disease activity during clinical trials.[80]

However, the NAS score was being widely misused to define and diagnose NASH and therefore in 2012 Bedossa *et al* created an algorithm to aid with the diagnosis of NASH known as the SAF score.[207] The score comprises of Steatosis, Activity (ballooning and lobular inflammation) and Fibrosis stage and it was proposed to be utilised to help discriminate between NAFL and NASH, as well as monitor disease activity during clinical trials.[80]

The criteria and grading tools used to describe NAFLD, NASH and Fibrosis have progressed over the years since their initial conception, below describes the histological components and their grading criteria as used in the Newcastle upon Tyne Hospitals, and therefore for this study.

1.9.2 Steatosis

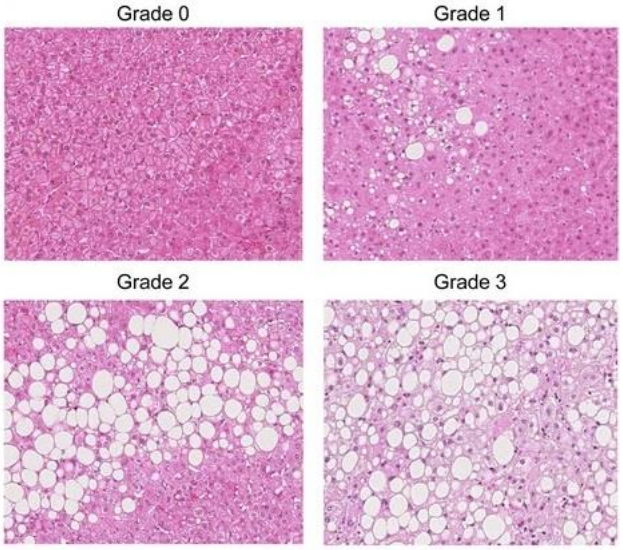
The term steatosis refers to the accumulation of both micro and macrovesicular hepatic fat (triglyceride) droplets.[208] The presence of fat droplets is graded depending on what proportion of hepatocytes are affected as identified at a low-medium power magnification.[209] Table 1.4 below displays the accepted criteria for steatosis grading and figure 1.4 shows the microscopic appearance matching each grade.

Table 1.4. NASH CRN criteria for steatosis grading in NAFLD histology.

Steatosis Grade	% of hepatocytes with fat droplets	Description
0	<5%	None/minimal
1	5-33%	Mild
2	34-66%	Moderate
3	>66%	Severe

(206, 209)

Figure 1.4 Microscopic appearance of steatosis grades based upon the NASH CRN criteria ranging from Grade 1, mild infiltration of fat droplets to hepatocytes, to Grade 3, micro and macrovesicular steatosis in more than two thirds of hepatocytes.



(205) *Image used with permission of publisher.

1.9.3 Steatohepatitis (NASH)

Non-alcoholic steatohepatitis (NASH) is defined as the presence of all three of: steatosis, hepatocellular injury, and lobular inflammation.

The hepatocellular injury is seen as balloon degeneration of hepatocytes; hepatocytes become enlarged, cytoplasmic changes occur and Mallory-Denk bodies (cytoplasmic inclusions that occur in ballooned hepatocytes) can be present.(208, 210) There is a recognised grading criteria for ballooning severity, as seen in table 1.5 below.

Table 1.5. NASH CRN criteria for ballooning grading in NAFLD histology.

Ballooning Grade	Definition	Description
0	None	No ballooned hepatocytes seen
1	Few/mild	Occasional ballooned hepatocytes present, near <50% of zone-3 areas or nodules
2	Prominent/many/ mod-severe	Clusters of easily identified ballooned hepatocytes or individual easily identified ballooned hepatocytes around >50% of zone-3 areas or nodules

(206, 209)

Lobular inflammation is characterised by the presence of inflammatory infiltrates such as lymphocytes, macrophages, eosinophils and occasional neutrophils.[205, 208, 209] This inflammatory process can occur intra-acinar (lobular) and around the portal tract and again a grading criterion is used to classify the extent of the inflammation as seen in table 1.6 below.

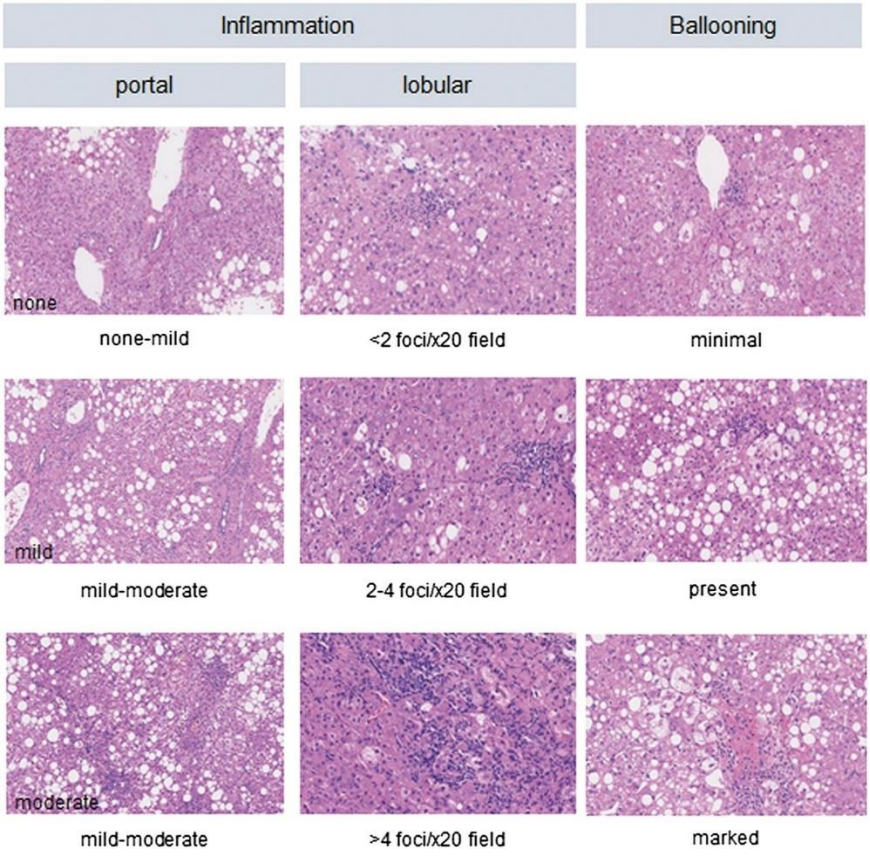
Table 1.6. NASH CRN criteria for inflammation grading in NAFLD histology.

Inflammation Grade	Definition	Description
0	None	Widely scattered foci of lobular inflammation (or very sparse infiltrates in a few portal areas)
1	<2 foci	Either occasional foci of lobular inflammation (>2 per x20magnification field) and/or mild portal inflammation
2	2-4 foci	Frequent foci of lobular inflammation (>2) or at least 2 portal areas with inflammation that replaces a portion of the matrix
3	>4 foci	Numerous foci of lobular inflammation with confluent inflammation in zone 3 or inflammation in the multiple portal areas that expands the portal tract matrix and spills out through the limiting plate

(206, 209)

Figure 1.5 below demonstrates the histological features of ballooning and inflammation as described above.

Figure 1.5. Histological features of ballooning and inflammation in NAFLD demonstrating mild to severe features of both such as balloon degeneration of hepatocytes and infiltration of inflammatory cells such as macrophages.



[211]*Image used with permission of publisher.

As described above, it is important to note that the definition and criteria of NASH has changed over the last two decades and thus some historical samples withing the Newcastle cohort may be graded according to more historical criteria.

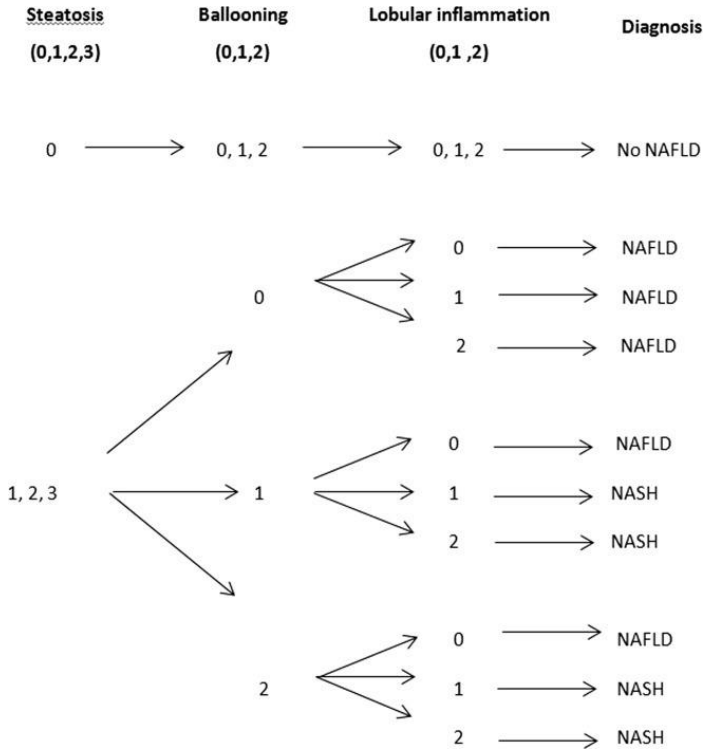
1.9.4 Markers of disease activity: NAS and SAF

As alluded to above, the NAS and SAF scores are used to describe and monitor disease activity in NASH.

The NAS score uses the above grading criteria for Steatosis (0-3), Ballooning (0-2) and Inflammation (0-3) to generate a total NAFLD Activity Score (0-8).[206] Over the years the ability of the NAS score to predict liver outcomes has been explored and while there have been some studies which suggest a NAS score ≥5 is predictive for a liver related death[97, 212], the general consensus is that NAS cannot reliably predict fibrosis and liver related outcomes.[94, 97, 99, 213]

The SAF score was created to help clarify the diagnosis of NASH with the use of the proposed algorithm as well as to generate an "activity score".[207] Bedossa and colleagues simplified the scoring for inflammation by making the maximum score 2 which represents >2 foci of inflammation per 20x field, and the ballooning definitions are slightly different than in the Kleiner system: grade 1 signifies the presence of clusters of hepatocytes which are of a similar size to normal hepatocytes but with a more rounded shape and pale, reticulated cytoplasm, and grade 2 signifies similar cytoplasmic features as grade 1 but in addition the hepatocytes are at least twofold larger than normal hepatocytes. Ballooning (0-2) and inflammation (0-2) results are then combined to create the Activity Score (0-4). Figure 1.6 below demonstrates the algorithm generated to facilitate in the diagnosis of NASH.

Figure 1.6. The diagnostic algorithm as developed by Bedossa et al to create the SAF score using a combination of Steatosis grade and Activity score [ballooning (0-2) plus inflammation (0-2]).



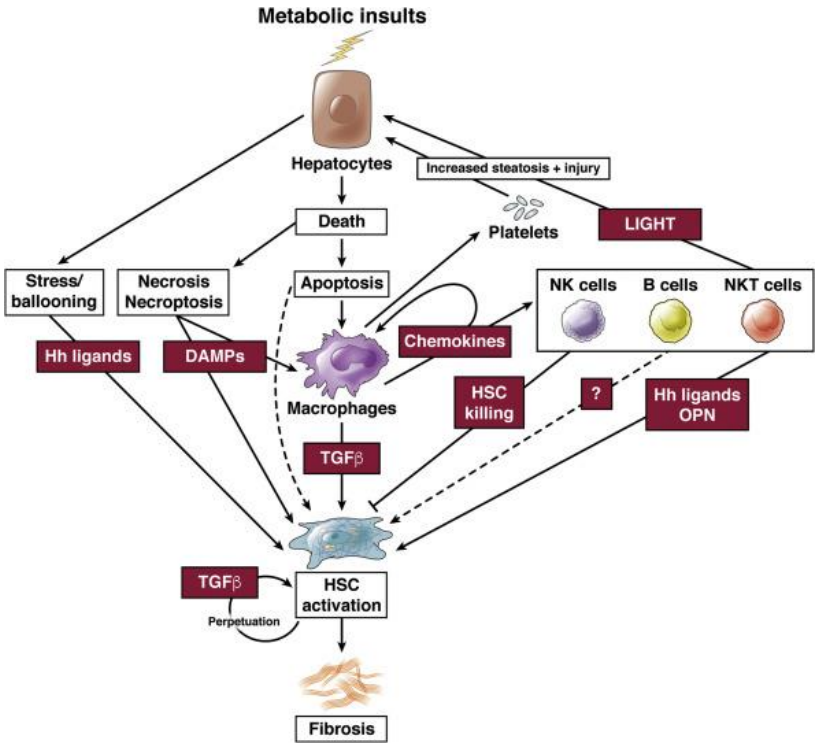
(207)

1.9.5 Fibrosis

The pathogenesis of fibrosis development in NAFLD is a complex process. Increased hepatic steatosis results in activation of a pro-inflammatory state with increase free fatty acids and oxidative stress. This results in hepatocellular injury through inflammation and ballooning of hepatocytes and further activates an inflammatory response with macrophages and further cytokines production. These factors result in activation of hepatic stellate cells which are the

main producers of extracellular matrix (ECM) producing fibroblast cells within the liver. This causes in an imbalance of ECM protein production-destruction, and thus accumulation of these proteins resulting in fibrosis.[209, 214] Figure 1.7 below demonstrates some of the factors involved in the pathogenesis of fibrosis in NASH.

Figure 1.7. Diagram demonstrating the mechanisms of action involved in the pathogenesis of fibrosis in NASH.



[214]*Image used with permission of publisher.

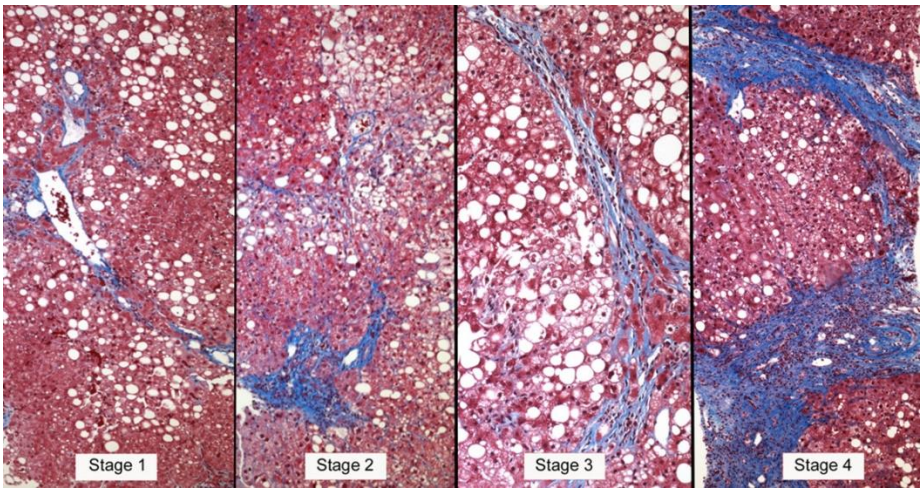
The development of fibrosis occurs in stages starting with it being focused in perisinusoidal or periportal zones and eventually progressing to bridging fibrosis and cirrhosis. The most validated and accepted way of scoring these stages is by the NASH CRN histologic scoring system[206], F0-4, and table 1.7 below demonstrates these stages and figure 1.8 demonstrates the histological appearance.

Table 1.7. Criteria for Kleiner fibrosis stages in NAFLD histology.

Fibrosis Stage	Definition	Description
0	None	
1 • A • B • C	 Mild perisinusoidal Moderate perisinusoidal Periportal/portal only	 Zone 3, mild fibrosis Zone 3, moderate fibrosis Periportal/portal fibrosis
2	Perisinusoidal & portal/periportal	Both periportal and zone-3 perisinusoidal fibrosis, without evidence of bridging fibrosis or cirrhosis
3	Bridging fibrosis	Presence of at least 1 definite abnormal fibrotic connection
4	Cirrhosis	Diffuse (≥50%) loss of architecture with regenerative nodules isolated by fibrosis

(206, 209)

Figure 1.8. Histological demonstration of the stages of fibrosis in NAFLD according to the NASH CRN staging system ranging from periportal zonal, progressing to bridging fibrosis and cirrhosis.



(145)*Image used with permission of publisher.

Over the years various studies have shown increasing fibrosis stage to be consistently associated with adverse clinical outcomes, liver related complications and mortality.(97, 99, 100)

1.9.6 Limitations to histology

There are limitations to the use of liver biopsy, which is why its use is reserved for select patients with diagnostic or disease staging uncertainty. A liver biopsy is an invasive

procedure and as such is associated with risks and complications for patients including pain, infection, and damage to visceral organs and its sequalae.[1-3, 215] Given the patchy nature of fatty liver disease and the fact a liver biopsy only samples 1:50,000th of the liver, there is also sampling error.[216] Finally inter-observer, and intra-observer, variability between different pathologists also has an impact on diagnostic reliability and studies have shown no improvement in concordance between members of the Pathology Committee of the NASH CRN in two separate studies over 15 years.[206, 217] Recent reports from Brunt *et al*[218] have highlighted significant differences in the identification of hepatocyte ballooning whilst Davison and colleagues[219] reported as many as 46.3% of patients included in a NAFLD clinical trial were reported to not meet the required histological inclusion criteria by a third of hepatology pathologists. These studies raise serious questions about the suitability of the utilisation of liver biopsy as an inclusion criterion or marker of change in clinical trials, which is the current standard practice. However, despite these limitations, liver biopsy remains the best-established way to differentiate NAFL from NASH and is used as the reference standard for staging fibrosis, i.e. the benchmark to which new non-invasive techniques are compared.

1.10 Associated clinical outcomes.

Due to the pathogenesis and co-morbidities associated with NAFLD, such as the MetS and T2DM, it is closely associated with other clinical conditions.

1.10.1 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is an endocrine disorder, affecting 2-26% of women of reproductive age[220], which is associated with insulin resistance and is considered the ovarian manifestation of the metabolic syndrome[73, 221, 222].

Given this association, a high proportion of patients with PCOS have hepatic steatosis, with one study demonstrating a 4 times increased rate of NAFLD in individuals with PCOS compared to healthy controls.[223]

Importantly, studies have also demonstrated that these individuals are also at a higher risk of developing NASH and advanced fibrosis [73, 224, 225], necessitating more aggressive screening and monitoring of disease stage in this group of patients.

1.10.2 Obstructive Sleep Apnoea

Obstructive sleep apnoea (OSA) is a sleep-related breathing condition characterized by episodes of complete or partial obstruction of the upper airway during sleep causing apnoeic episodes.[226] It is directly related to obesity and has been independently linked to all the components of the metabolic syndrome.[227] OSA is therefore also independently associated with NAFLD. Recent studies have shown as many as 85% of patients with severe OSA have steatosis and an increased risk for the development of NASH and fibrosis, with one study demonstrating 26% of those with severe OSA also having fibrosis.[228, 229] The chronic intermittent hypoxia from apnoeic episodes is believed to contribute to the pathogenesis of NASH through hepatocellular oxidative stress.[230, 231]

1.10.3. Chronic Kidney Disease

Chronic kidney disease (CKD) is defined by the National Institute for Health and Care Excellence (NICE) as a reduction in kidney function or structural damage (or both) present for more than 3 months, with associated health implications.[232-234] It is estimated to affect 10% of the UK population[235] and is strongly associated with features of the metabolic syndrome such as T2DM, HTN and obesity.[236] Given these common risk factors, patients with NAFLD have higher rates of CKD than the general population with a metanalysis suggesting a two-fold increased risk of CKD in the NAFLD population over time[236].

1.10.4. Cardiovascular disease (IHD and Stroke)

The risk factors for the development of cardiovascular disease (CVD), which by definition includes ischaemic heart disease and stroke, have significant overlap with the risk factors for NAFLD including abdominal obesity, dyslipidaemia, hypertension, and diabetes.[237] As a result, patients with NAFLD have an increased risk of CVD. There is a growing body of evidence to suggest that patients with NAFLD are at an increased risk of CVD independently of these shared risk factors, however this remains controversial.[12, 238, 239] The mechanism for this is not well understood but may be due to oxidative stress and low-grade inflammation associated with NASH, and altered gut microbiota.[9, 237, 239]

One recent meta-analysis found the risk of atrial fibrillation (AF) is doubled in patients with NAFLD, independent of other known risks for AF[240], another demonstrated a significant relationship between NAFLD and the development of diastolic dysfunction[241] and the link between the increased prevalence of atherosclerosis and NAFLD is also well documented.[242, 243] Most importantly however, cardiovascular disease has been widely shown to be the

leading cause of mortality in patients with NAFLD, accounting for 25-60% of deaths in this group.[50, 93, 244, 245]

1.10.5 Extra-Hepatic Malignancy

In recent years there has been a recognition of the increased prevalence of extra-hepatic malignancy in patients with NAFLD, and cancer is the second most common cause of mortality in these patients.[9] The most frequently reported site of extra-hepatic malignancy is gastrointestinal, in particular colorectal, but there is also an association with oesophageal, breast, gynaecological and prostate.[246, 247] This increased risk was initially thought due to the known association with obesity and cancer but there have been recent studies which suggest that NAFLD may provide a risk of malignancy independent of obesity due to inflammatory processes associated with NASH.[246, 248] Overall, meta-analyses have shown a lifetime two-fold increased risk in the development of all cancers in patients with NAFLD.[239, 247, 249]

1.11 Mortality

Several studies have been undertaken comparing patients with NAFLD to a reference population, and these have shown that a diagnosis of NAFLD is associated with an overall increased mortality.[80, 93, 97, 250] In a meta-analysis Musso *et al*[251] found an increased risk of death in patients with NAFLD (odds ratio 1.40: 95% CI 1.23-1.60) and in a recent population-based study Simon and colleagues[252] found that compared to controls patients with NAFLD had an adjusted hazard ratio of 1.93 (95% CI 1.86 – 2.00) for death. The most common reported causes of death in patients with NAFLD are cardiovascular disease (8.1% - 61.5%)[36, 92, 93, 99, 253, 254], malignancy (5.4% - 32.4%)[36, 92, 99, 252-254] and liver disease related (9% - 85%)[36, 92, 97, 99, 253, 254]. These studies are all highly selective, for example Vilar-Gomez *et al*[253] were following up a group of patients with advanced fibrosis at the time of selection (F3-4) and therefore found a high proportion of their cohort died as a result of the underlying liver disease. There have been very few population-based studies to demonstrate a mortality in a less selective cohort of NAFLD patients, but in the recent population-based study by Simon *et al*[252] 14.4% of patients with biopsy confirmed NAFLD died of liver disease (aHR 18.15), 32.4% of extra-hepatic malignancy (aHR2.16) and 28.9% cardiovascular disease (aHR 1.35).[252]

As described above there are many studies that have detailed long-term outcomes in NAFLD cohorts, with varying diagnostic criteria and levels of detail described. Table 1.8 below summarises some of the key studies that explore the long-term outcomes and mortality of NAFLD patients. These studies were chosen in particular as they were felt to have similar eligibility criteria to the Newcastle long-term outcome study, i.e., histologically diagnosed NAFLD, and also provided detail about outcomes of interest including of cause of death, prevalence of co-morbidities and incidence of clinical outcomes of interest such as development of hepatocellular carcinoma.

Table 1.8. Summary table of literature reporting the long-term outcomes in NAFLD, chosen for review due to similar qualities to this thesis from the Newcastle NAFLD cohort.

Author, Year, Location	No of pts	Diagnostic inclusion criteria	Follow-up duration (mean)	Baseline demographics	No. of deaths[#] (%)	Cause of death				Reported outcomes of interest
						Liver[#]	CVD[+]	Cancer	Other	
Adam,[255] 2005, USA	435	US or LBx, all stages NAFLD	7.6 yrs	49% male 49 years 92% Caucasian 26% T2DM 71% Obese 36% HTN	53 (12.6%)	7 (13%)	14 (27%)	15 (28%)	17 (32%)	SMR* 1.34 Age HR 2.2 T2DM HR 2.6 Cirrhosis HR 3.1 IHD HR 1.1
Ekstedt,[93] 2006, Sweden	129	LBx, all stages NAFLD	13.7 yrs	67% male 51.0 years 8.5% T2DM 29% Obese 72% HTN 11% CVD 55% NASH	26 (20.2%)	2 (7.7%)	16 (61.5%)	5 (19.2%)	3 (11.5%)	Reduced survival with NASH (p0.01) 2.3% developed HCC 41% had progression of fibrosis

Bhala,[256] 2011, Multi-national (USA, UK, Italy, Australia)	247	LBx, F3-4. HCV controls.	7.2 yrs	39.7% male 54.7 years 91.5% Caucasian 50.6% T2DM 44.1% HTN 12.1% CVD	33 (13.4%)	14 (42.4%)	1 (3.0%)	4 (12.1%)	14 (42.4%)	19.4% developed decompensation Overall mortality: Age aOR 1.55 Liver mortality: F4 aOR 10.43 CV event: T2DM aOR 10.43
Stepanova,[36] 2013, USA	289	LBx, all stages NAFLD	12.5 yrs (med)	39.4% male 78.6% Caucasian 46.0% Obese 26.0% T2DM 59.2% NASH	115 (39.8%)	30 (26.1%)	32 (27.8%)	18 (15.7%)	35 (30.4%)	Age aHR 1.07 T2DM aHR 2.09 NASH aHR 1.13 Liver specific: NASH aHR 9.16 T2DM aHR 2.19 Cardio specific: T2DM aHR 1.71

Ekstedt,[97] 2015, Sweden ^	229	LBx, all stages NAFLD. Population controlled	26.4 yrs	66% male 48.8 years 26% Obese 14% T2DM 57% HTN 9.7% IHD	96 (41.9%)	9 (9%)	41 (43%)	22 (23%)	24 (25%)	Overall mortality HR 1.29 Cirrhosis HR 3.2 F3/4 HR 3.3 CVD HR 1.55 HCC HR 6.55
Sebastiani,[254] 2015, Canada	148	LBx, all stages NAFLD, all NASH	5 yrs (med)	69.6% male 49.5 years 33.1% T2DM 39.2% HTN 28.4% MetS 33.8% F3/4	11 (7.4%)	5 (45.5%)	4 (36.4%)	0	2 (18.2%)	APRI >1.5 aHR 5.02 FIB-4 >3.25 aHR 6.33 NFS >0.676 aHR 11.9 F3/4 aHR 3.14

Angulo,[99] 2015, multi-national (USA, UK, Australia, Denmark, Iceland, Thailand)	619	LBx, all stages NAFLD	12.6 yrs	37.5% male 49 years 88% Caucasian 37.5% T2DM 30.7% HTN 28.9% NASH 11.5% F3/4	193 (31.2%)	18 (9.3%)	74 (38.3%)	36 (18.7%)	65 (33.7%)	26 (4.2%) developed decompensation All-cause mortality: F4 aHR 6.35 Age aHR 1.07 T2DM aHR 1.60 Smoking aHR 2.62 Liver mortality: F3/4 aHR 85.79
Hagstrom,[98] 2017, Sweden~	646	LBx, all stages NAFLD. Population controlled	19.9 yrs	62.2% male 48.2 years 14.4% T2DM 30.3% HTN 66.4% NASH 12.0% F3/4	214 (33.1%)	17 (7.9%)	79 (36.9%)	55 (25.7%)	63 (29.5%)	Overall mortality HR 1.14 F3 aHR' 1.76 F4 aHR' 3.75 NASH not significant 11.6% fibrosis progression

Younossi,[257] 2017, USA	209	LBx, all stages NAFLD.	12.5 yrs (med)	37.8% male 48.7 years 75.1% Caucasian 48.8% Obese 20.6% T2DM 62.7% NASH	64 (30.6%)	18 (8.6%)	22 (10.6%)	-	-	Liver specific mortality NASH aHR 9.9 Steatofibrosis aHR 6.7 Bridging fibrosis/ cirrhosis aHR 17.5 CVD death not associated with above
Vilar-Gomez, [253] 2018, multi-national (Spain, Hong-Kong, Cuba, Australia)	458	LBx, F3/4 NAFLD	5.5 yrs	48% male 55.9 years 81% Caucasian 67% T2DM 61% HTN 9% IHD	74 (16%)	68 (91.9%)	3 (8.1%)	2 (2.7%)	-	All-cause mortality Cirrhosis HR 5.99 T2DM HR 3.33 Varices HR2.19 Steatosis <33% HR 2.56

Simon,[252] 2020, Sweden	10, 568	LBx, all stages, population matched controls	14.2 yrs	55.2% male 52.0 years 4.4% Obese 11.2% T2DM 9.8% HTN 2.8% MetS 20.1% IHD	4338 (41.0%)	599 (13.8%)	1199 (27.6%)	1343 (31.0%)	1008 (23.2%)	Compared to controls: Mortality aHR 1.93 NAFL aHR 1.71 F0 NASH aHR 2.14 F1-3 aHR 2.44 Cirrhosis aHR 3.79 CVD death aHR 1.35 Cancer death aHR 2.16
[#] Mortality includes OLTx. [+]Cardiovascular disease including IHD and stroke. *SMR= standardised mortality ratio. ^Includes patients from Ekstedt 2006 paper. ~Includes patients from Ekstedt 2006 and 2015 papers. ‘Controlled for age, sex and T2DM.										

1.12 Management

With the increasing burden of NAFLD on healthcare over the last two decades there has been a significant effort to identify potential treatments. Currently there remains no approved therapies for NASH either by the FDA or the European Medicines Agency (EMA) and as such the focus of management is currently more holistic and centred around the reduction of metabolic and cardiovascular risks associated with disease progression and mortality.[1-3, 9, 124, 161, 238]

1.12.1 Lifestyle interventions- Diet

Lifestyle interventions with the goal of weight loss is currently the mainstay of management for patients with NAFLD.[1-3, 9, 161] There have been many studies over the last 30 years that have shown a reduction in total body weight can improve hepatic steatosis. Studies initially described this improvement by a reduction in aminotransferases and reduced steatosis on abdominal ultrasound[258-260] and MR spectroscopy.[131, 137] The reduction in hepatic steatosis ranged from 20-81% and was proportional to the intensity of lifestyle change, an overall total weight loss of 5-10% was generally required for an improvement in MR appearance.[261-263]

This improvement associated with weight loss was also seen histologically. One small study of 31 obese patients allocated participants to either intense lifestyle changes with diet, behaviour modifications through weekly therapy sessions and an exercise programme with the goal of 200 minutes activity per week, or a basic education programme.[264] Over 48 weeks of follow-up the intervention group were found to have achieved 9.3% weight loss vs 0.2% in the control group. Most importantly follow up histology showed significant improvement in steatosis, inflammation, ballooning, and NAS score in those who had achieved ≥7% weight loss. Vilar-Gomez and colleagues[265] undertook a similar study with 261 participants given 12 months of a calorie-controlled diet, exercise programme of 200 minutes/week and therapy sessions every 8 weeks to promote adherence. At the end of the study 25% of participants had resolution of NASH, 47% had a reduction in the NAS score and most importantly 19% showed a reduction in fibrosis stage on repeat histology. This was directly correlated to the amount of weight lost, those who lost ≥10% of their total body weight all had a reduction in NAS, 90% had resolution of NASH and 45% had fibrosis regression.[265]

There have been many different diet methodologies proposed to aid weight loss in the NAFLD community. The Mediterranean diet is one approach which has shown to successfully improve hepatic steatosis and is also well tolerated with greater adherence than others, and as such is recommended by EASL.[1] This diet focuses on emulating the cultures of the southern Mediterranean countries such as Italy and Greece where the diet is high in fruits, vegetables, wholegrains, cereals, nuts, pulses, seeds, and fresh fish. It is not a low-fat diet but focuses on the use of healthier monounsaturated fat sources such as olive oil, nuts and oily fish instead of saturated sources such as dairy and red meat.[266, 267] Studies have shown this diet is easier to adhere to in the long-term due to the variety of foods and flavours this includes, but one of the major downsides is the cost implications of buying fresh produce.[51, 268, 269]

Other diet methods trialled in the NAFLD cohort include intermittent fasting, very-low-calorie-diet (VLCD), low fructose intake, low-fat high-carbohydrate, and calorie restriction with a high fibre intake to name a few. Each method has different pros and cons, but a recent meta-analysis of different approaches has suggested that ultimately weight loss is proportional to the calorie deficit regardless of the method used to achieve this, and long-term adherence or lifestyle change should be the target to achieve and maintain this weight loss.[1, 3, 270-273]

1.12.2 Lifestyle interventions- Exercise

In recent years evidence has shown that patients with NAFLD in general are more sedentary than the general population and therefore encouraging an increase in physical activity levels is also an important aspect of the management of NAFLD.[238, 274, 275] Several studies have investigated the effect of exercise alone on NAFLD and one randomised control trial involving 24 patients with NASH and sedentary lifestyles found that in those who were given a structured exercise plan over 12 weeks there was a significant reduction in hepatic triglyceride content, visceral adiposity and serum triglyceride levels. Participants were encouraged to maintain their weight during this study, demonstrating the independent positive effects of exercise.[129]

As with diet, there are debates as to the best method of exercise for patients with NAFLD such as resistance training vs aerobic activity. One meta-analysis has shown that both these methods of exercise reduce hepatic steatosis in patients with NAFLD and have different benefits.[276] Aerobic exercise for example has a greater energy consumption and overall

benefits for cardiorespiratory fitness, but resistance training is also beneficial for cardiorespiratory function and may be better tolerated in patients who are unable to tolerate aerobic exercise. It is therefore recommended that patients with NAFLD participate in 150-200 minutes per week, in 3-5 sessions of moderate-intensity exercise of their choosing in order to encourage adherence and long-term effects.[1]

This recommendation for exercise is in conjunction with a healthy diet as studies have found the combination to be optimal at achieving and maintaining weight loss.[1-3, 161, 169]

It is important to recognise that achieving and sustaining weight loss is difficult and for many patients there are barriers to attaining this goal including financial constraints for expensive diets, physical limitations to exercise and poor education surrounding health choices.[9] These are often underpinned by health inequality issues which affect all areas of the world, not simply developing countries.

1.12.3. Weight loss interventions

The use of agents to assist in weight loss such as Orlistat, a gut lipase inhibitor, has been explored in the NAFLD population. Orlistat reduces the absorption of dietary fats and is recommended to be used in conjunction with a calorie-controlled diet.[277, 278] The early evidence for its use suggested patients benefited from significant weight loss with associated improvement in liver blood tests and hepatic steatosis, inflammation, and fibrosis.[279, 280] However other studies have reported no additional benefit from its use with regards to weight lost or histological improvement of NAFLD.[278, 281] The side effect profile of the drug is also not generally well tolerated and for these reasons it is rarely used as a treatment for NAFLD nowadays.

Recently NICE have approved the use of Semaglutide as a weight loss aid for patients in the U.K. under specialist weight management services with a BMI ≥35.0 kg/m^2 and one weight related co-morbidity.[282] Semaglutide is a glucagon-like peptide 1 (GLP-1) agonist originally developed for the management of diabetes which has been shown to lower blood glucose levels by stimulating insulin and inhibiting glucagon secretion.[283] It has also been shown to reduce appetite and result in a reduction in caloric intake. The combination of these mechanisms of action has been shown to result in sustained weight loss and improvement in glycaemic control over many studies.[283, 284] In more recent years its use has been trialled in

the management of NAFLD and been linked to a reduction in liver enzymes as well as some histological improvement.[285, 286]

Bariatric surgery is not currently indicated as a first line therapy for NAFLD, but it is a viable option to help achieve significant and more sustainable weight loss in those who are morbidly obese.[1-3, 161] Early studies indicated that all bariatric procedures were associated with a reduction in steatosis, ballooning, and NASH post procedure,[287, 288] however a Cochrane review in 2010 raised questions about the effectiveness of this treatment given a lack of randomised trials.[289] In 2020 Lassailly and colleagues[290] published a prospective study of 180 severely obese patients with biopsy proven NASH who underwent bariatric procedures and then 5-year follow-up with repeat liver histology. This found resolution of NASH by 1 year in 85%, and importantly also demonstrated a progressive reduction in fibrosis over this time period in 70.2% of patients, with complete resolution in 56%. However, further studies are still required to fully comprehend the optimal procedures and concurrent lifestyle interventions for the NAFLD cohort.[238]

1.12.4 Optimizing co-morbidities.

Given the strong associations between NAFLD and co-morbidities such as hypertension, dyslipidaemia, and diabetes, one of the key management strategies for NAFLD is to screen patients regularly for the presence of these conditions and optimise their management.

In the U.K. the first line treatment for type 2 diabetes is Metformin. This medication has been found to aid in weight loss and improve all diabetes related clinical outcomes including microvascular disease, large vessels disease and cardiovascular mortality.[291] There have been several studies undertaken to explore the benefit in NAFLD and whilst it does not appear to confer any benefit in improving liver blood tests or histology,[3, 271] there is some evidence to suggest it's use results in a reduction in the incidence of HCC and extra-hepatic malignancy of up to 7% in patients with NASH.[278, 292] For this reason, it remains a reasonable first line treatment for T2DM in the NAFLD cohort. In those with poor diabetic control despite Metformin treatment the addition of a second agent would be recommended. In particular GLP-1 analogues, such as Liraglutide, or SGLT-2 inhibitors, such as Empagliflozin, have been shown to also aid weight reduction and improve hepatic steatosis and inflammation.[1, 3, 9, 278, 293, 294]

Dyslipidaemia is another important co-morbidity which should be aggressively managed in patients with NAFLD. Historically there has been concern about the use of statins for lipid-management in the NAFLD cohort due to reports of hepatotoxicity. However, this a rare occurrence and statins have been shown to be not only safe in this cohort but also improve overall mortality.[295, 296] In the U.K. it is recommended that patients with NAFLD have a QRISK-3 score calculated (a tool developed to identify patients at risk of cardiovascular disease) and statin treatment offered to individuals with a >10% risk of a cardiovascular risk in the next 10 years.[169, 278, 294, 297]

Other important risk modifying factors which should be considered in patients with NAFLD include smoking cessation and alcohol consumption advice. As already discussed, alcohol intake of >30g/day (men) and >20g/day (women), excludes a sole diagnosis of NAFLD. Data have been conflicting on whether consumption of alcohol within recommended limits is harmful in patients with NAFLD. It is important to advise patients about the potential additive effect of alcohol consumption and metabolic associated steatosis promoting fibrosis progression. However further work is required to fully understand the risks of even "safe" alcohol consumption in patients with NAFLD.[3, 11, 294]

1.12.5. "Off-label" recommendations for NAFLD and emerging therapies

As mentioned earlier, there are no medications currently licenced for the treatment of NAFLD. One of the recognised limitations in the development of treatments for NAFLD is the accepted endpoint design of NASH clinical trials is based upon clinical and specific histological improvement, for example the resolution of NASH with no worsening of fibrosis score, or the improvement in fibrosis score. Given the sampling errors and inter-observer variability already discussed, in addition to the cost and safety aspect of serial liver biopsies this has had a significant impact on the development of drug treatments in NAFLD.[298]

However, there are several medications which are available "off-label" that have been found to have varying degrees of success in NAFLD patients. Table 1.9 below provides a list of the most widely available and discussed drug therapies.

Table 1.9. Summary of drugs currently used and emerging treatments in NAFLD including drugs currently licenced for diabetes and weight management. [1-3, 9, 238, 278]

Drug Class	Example	Liver effect	Other benefit	Side effect/ Safety concern	Ref
Antioxidant	Vitamin E	Improves NASH and fibrosis May prevent decompensation and reduce mortality	n/a	↑ risk of bleeding Prostate Ca Haemorrhagic stroke All-cause mortality	(299-303)
Thiazolidinedione (PPAR agonist)	Pioglitazone	Improved inflammation and fibrosis	Improved diabetic control or may prevent T2DM	Weight gain Osteoporosis Fluid retention Bladder cancer CCF	(301, 304-307)
GLP-1 agonist	Liraglutide, Semaglutide	Improves steatosis and inflammation NASH resolution	↓ weight Improved diabetic control ↓cardiovascular event	GI upset Dyspepsia AKI	(286, 308-311)
SGLT2 inhibitors	Empagliflozin, Dapagliflozin	Improves steatosis and inflammation Improves liver enzymes	Improved diabetic control ↓ weight Renoprotective ↓cardiovascular event	Genitourinary infections AKI Euglycaemic ketoacidosis	(312-315)
DPP-4 inhibitors	Sitagliptin	Improved steatosis Reduction in NAS scores	Improved insulin sensitivity	Nasopharyngitis Bullous Pemphigoid	(197, 316, 317)
ACE inhibitors	Ramipril	Anti-fibrotic effect Possible reduced HCC	Hypertension control Cardioprotective	Renal impairment Hyperkalaemia	(318-320)
Farnesoid X receptor agonist (FXRs)	Obeticholic acid	Antifibrotic effect	n/a	Elevated LDL Pruritis	(321, 322)

1.13 Future

In recent years, NAFLD has become a leading cause of chronic liver disease worldwide. With rising obesity rates, prevalence of NASH is likely to continue to rise, resulting in high rates of advanced fibrosis/cirrhosis and associated complications. This will come with significant economic and healthcare demands.[72]

In an effort to encourage healthcare policies and strategies to meet the rising demands, various clinical models have been developed to help predict disease burden and healthcare needs. Estes and colleagues[82, 323] have used Markov modelling from countries with available data to forecast the prevalence of NAFLD globally. They predict within Europe the United Kingdom will have the largest increase in cases, projecting an increase of 20.2% by 2030.[323] In the United States it is predicted that NAFLD prevalence will rise by 18.3% which would account for over 15 million new cases by 2030. However, what is most concerning about these predictions is the associated morbidity and mortality that will accompany the rising rates. Liver related mortality is forecast to increase by 159% in this time.[323] A similar study, also using Markov modelling, demonstrated the prevalence of NASH in the US was predicted to rise by 63%, leading to a 180% increase in decompensated cirrhosis cases and 137% increase in the incidence of HCC over a 15-year period from 2015-2030.[82]

Although significant progress has been made in understanding the natural history and progression of NAFLD since it was first reported as an independent cause of chronic liver disease by Ludwig and colleagues,[202] we are still not reliably able to predict and prevent disease progression in patients.[9] For this reason, ongoing study into modifiable risk factors such as environmental influences and the impact of other co-morbid conditions and medications, as well as the further evaluation of tools which may predict disease progression or HCC development are of such importance.

1.14 Chapter summary and study objectives.

Non-alcoholic fatty liver disease has become a leading cause of chronic liver disease worldwide. Increasing prevalence, particularly in Western countries such as the United Kingdom, has resulted in a significant increase in morbidity and mortality associated with NAFLD. Heterogenicity of outcomes within NAFLD has posed a challenge in the long-term management, and further studies exploring the natural history and outcomes of these

patients are required to improve monitoring and treatment in a safe, cost-effective, and practical manner. This study aims to examine a large, single centre, U.K. based NAFLD cohort with a long follow-up period of up to 35 years with the goal of identifying factors which may be prognostic of adverse outcomes such as the development of cirrhosis and complications such as HCC, as well as mortality.

Chapter 2: Research Aims and Study Methodology

2.1 Research Aims

This study aims to gain a further understanding of the natural history of Non-Alcoholic Fatty Liver Disease by assessing a large, single-centre cohort of patients with a diagnosis of NAFLD over a 30-year period. The outcomes of these patients will be explored, specifically the frequency of significant clinical outcomes such as progression to cirrhosis, development of hepatocellular carcinoma, death, liver transplantation and cardiovascular events.

2.1.1 Research Questions for this study

1. What are the baseline and final event characteristics of the Newcastle NAFLD cohort?

2. What is the frequency of significant clinical events in patients with NAFLD?

3. What factors predict significant clinical events in patients with NAFLD?

4. How accurately do the currently used prognostic tools predict outcomes?

2.2. Ethical approval and research governance

2.2.1 Ethics

Ethical approval was sought from the Research and Ethics Committee and initially granted on 23rd April 2019 from North East- Newcastle & North Tyneside 1 Research Ethics Committee.

A substantial amendment was submitted on 24th July 2019 following review by the Confidentiality Advisory Group (CAG) committee, and approval received on 25th September 2019.

2.2.2 Confidentiality Advisory Group

At the same time as the above ethics application, a submission was made to the Confidentiality Advisory Group to access data on non-consenting patients. CAG reference 19/CAG/0095. This application initially sought approval to obtain “opt-out” consent in the group of patients that could be contacted by letter, and approval to access data without consent in those we could not contact. Due to changes in General Data Protection Regulation (GDPR), the CAG team no longer supported the process of “opt-out” consent and therefore we received a “deferred” outcome on 10th July 2019.

Advice and guidance was sought from the CAG team regarding the best way to proceed and the recommendation was to re-apply for CAG approval on the grounds of obtaining data without individual permission but based on the principal of "greater good". On discussion with the CAG team, it was felt the outcomes of this study would increase understanding of NAFLD which, as discussed in chapter 1, has been increasing in prevalence and associated with an increasing burden on healthcare in the U.K. It was therefore felt access to these data would be in the best interest of the general population and on this grounds would be reasonable to access without specific written consent when it was unable to be obtained. As part of their approval the CAG team required suitable publication of the study intentions to access this data. This was done by advertising with posters in public spaces patients may see, for example the hospital corridors, or by posting information sheets to those patients whose details we were able to obtain. The patient information sheet and posters used are demonstrated in Appendices A, C and D.

The CAG team also wished to see how the data was expected to be accessed and stored and this was demonstrated in a flow diagram which can be seen in appendix E.

Following a change in methodology, and the resultant substantial amendment, the application was reconsidered and given "fully supportive" outcome under Regulation 5 of the Health Service (Control of Patient Information) Regulations 2002, section 251, to process confidential patient information without consent on 9th October 2019.

2.2.3 Health Research Authority Approval

The HRA and Health and Care Research Wales (HCRW) granted initial approval for the study on 10th July 2019. REC reference 19/NE/0092.

Following the favourable CAG and REC outcomes, the HRA granted approval for the study on 11th October 2019.

2.2.4 Caldicott Application

Newcastle upon Tyne Hospitals NHS Foundation Trust granted Caldicott approval for the study on 3rd January 2019. Reference ID: 6945.

2.3 Study design

2.3.1 Overview

As a tertiary liver centre, the Newcastle-upon-Tyne Hospitals hepatology department has performed diagnostic and staging liver biopsies on patients with liver diseases, including NAFLD, as a matter of routine clinical care for decades. As part of this routine care, a clinical database initially set up by Professor C Day has been kept since the 1980s for patients diagnosed with NAFLD from liver biopsy. This cohort was the basis for this study, and we aimed to collect up-to-date clinical information on these patients to answer the research questions stated above. A number of patients were lost to follow-up since their initial liver biopsy and the ethical considerations regarding using their data is discussed below.

2.3.2 European NAFLD Registry

The European NAFLD Registry is an international, prospectively recruited observational cohort study that aims to establish a large, highly phenotyped patient cohort with matched bioresource.[324] This study was developed by Professor Anstee and his research team at Newcastle University and Newcastle upon Tyne Hospitals in 2010, building upon the database begun by Professor Day and his research team. This is now part of the LITMUS Consortium, which recruits patients from various centres across Europe and USA.

As a result, a large proportion of patients who are in the historical clinical database, and remain under the care of the Newcastle Hospitals, have been subsequently recruited and consented into the European NAFLD Registry. Therefore, a proportion of the study population has come from the registry.

2.3.3 Recruitment and consent process

The informed consent for patients who were already part of the European NAFLD Registry allows use of their linked-anonymous data for research purposes related to their underlying condition (NAFLD). We were therefore able to access the existing data for these patients without seeking further written consent.

In those patients who were not consented into the registry we sought REC and CAG approval to access relevant clinical data without written consent, as detailed above. In this group of patients, where we were able to, a letter was written to inform them about the study, its aims and how their data may be used. This letter also contained information for them to request not to be included in the study. In order to raise awareness of the study for those

who we were not able to contact, we advertised the study within the hospital on notice boards, on the trust website and using our local liver patient support group, LIVErNORTH.

2.4 Eligibility Criteria

2.4.1 Inclusion Criteria

In order to be eligible for inclusion in the study, patients had to fulfil the following criteria:

1. Clinically suspected NAFLD with either a) a historical liver biopsy confirming NAFLD, b) patient undergoing a liver biopsy with biochemical/radiological findings consistent with NAFLD or c) radiological evidence of cirrhosis plus the presence of ≥ 2 features of the metabolic syndrome and no exclusion criteria.
2. Non-alcoholic fatty liver disease as confirmed on liver biopsy available at Newcastle upon Tyne Hospitals between 1990-2018.
3. Age >18 years old at the time of consent.

2.4.2 Exclusion Criteria

Patients could not be included if:

1. Alternative cause for steatosis was demonstrated on liver biopsy such as excessive alcohol consumption (sustained consumption of >14 units/week in the past 5 years) or use of steatogenic drugs including, but not limited to; Methotrexate, Amiodarone or Tamoxifen.

2.4.3 Exceptions to histology proven NAFLD

A small number of patients (<5%) included in the study had advanced disease by the time of referral to the Newcastle Hospitals and therefore a liver biopsy was not clinically indicated as part of routine care. These patients did not have histology available but were well characterised NAFLD patients and therefore included in the study.

2.5 Data management

2.5.1 Data collection

Data collected for the study was recorded on the European NAFLD Registry database. Patients consented into the registry had prospective data collected by the Hepatology Research Team at specific clinical events according to protocol including baseline/enrolment,

annual review, liver biopsy, significant clinical event (such as hospitalisation for illness) and death. The remaining patients had retrospective data collection entered into the registry recorded as “medical notes review”.

2.5.2 Enrolment & Baseline Visit

Upon inclusion in the registry eligibility criteria were recorded along with routine clinical data, as discussed below. There were several additional initial laboratory investigations also recorded at baseline in order to confirm eligibility and exclude alternative causes of liver disease. These included: Ferritin, Transferrin saturations, Urate, CRP, IgG, IgM, IgA, ANA titre, AMA titre, aSMA titre, HBV sAg, HBV cAb and HCV Antibody.

A more in-depth social history was also taken at baseline including details of education level achieved, employment details, smoking history, illicit drug use and family history.

2.5.3 Routinely collected data.

After the recruitment data entry, follow-up data were recorded at the time of annual review, repeat liver biopsy or significant clinical event including hospitalisation for any reason. In those who had become lost to follow-up an up-to-date medical review record was created for this study. Each entry required confirmation of ongoing eligibility for inclusion to the registry. Data recorded at these events is detailed below in table 2.1.

Table 2.1. Routinely collected data throughout study.

Category of data	Variable recorded
Basic data	Sex Age Weight (kg), and BMI Waist circumference (cm) Hip circumference (cm) BP recording (mmHg)
Blood results	Full blood count (Hb, Platelet, Clotting, Fibrinogen) Biochemistry (Albumin, Bilirubin, AST, ALT, ALP, GGT, Creatinine) Other (HbA1c, fasting glucose, total cholesterol, HDL, LDL, Triglycerides, TSH)
Radiology	AUSS Fibroscan™
Prognostic Tools	NAFLD Fibrosis Score FIB-4 Score MELD Score

Medical History -diagnosis -date of diagnosis	T2 DM HTN Dyslipidaemia; High triglycerides and High Cholesterol Hypothyroidism OSA Depression Gallstones; if yes cholecystectomy details also recorded CKD IHD AF CVA CCF PVD Cirrhosis: if yes decompensation and HCC details also collected Malignancy; if yes type of cancer Bariatric surgery Additional medical history
Concomitant medication -drug class -drug dose -start date	Anti-hyperglycaemic drugs Lipid lowering drugs Anti-hypertensive drugs Insulin "Other" medications Complimentary/alternative medications
Social Factors	Alcohol consumption: current and previous Caffeine consumption: tea, coffee, soft drinks

2.5.4 Liver biopsy data

Liver biopsy data were recorded in the registry at the time of baseline, and any subsequent biopsies performed. Details including the route of biopsy, i.e., transcutaneous, were routinely recorded along with the length (mm), number of fragments taken and hepatic venous pressure gradient (HVPG) if the biopsy was trans-jugular in route.

Clinical data from the time of the biopsy were recorded as in table 2.1 above. The histological report of the biopsy was recorded as detailed in table 2.2 below.

Table 2.2. Histological data collected at liver biopsy entry.

Histological Feature	Result
	General diagnostic category of NAFLD
Steatosis	Kleiner/SAF Steatosis Score (0-3) Steatosis % Steatosis Type
Ballooning	Kleiner/SAF Ballooning Score (0-2)
Inflammation	Kleiner Lobular inflammation Score SAF Lobular Inflammation Score Portal Inflammation Score
Fibrosis	Kleiner/SAF Fibrosis Stage (0-4) Expanded Fibrosis Stage (0-6)
Activity Scores	NAS- score (0-8) SAF Activity Score (sum of SAF inflammation and ballooning)

2.5.4 Death Record

Patients who died during the follow-up for the study had a record of death created in the registry database. A number of patients had died in the Newcastle Hospitals, and therefore the cause of death documented in the clinical notes was recorded in the registry. In those patients who had died outside the Newcastle Hospitals, the cause of death was not available in the medical notes, and an application was made to the appropriate local authority to obtain the death certificate in order to record the cause of death.

The cause of death was recorded as per the certificate; Ia, b, c, II.

2.5.4 New data points

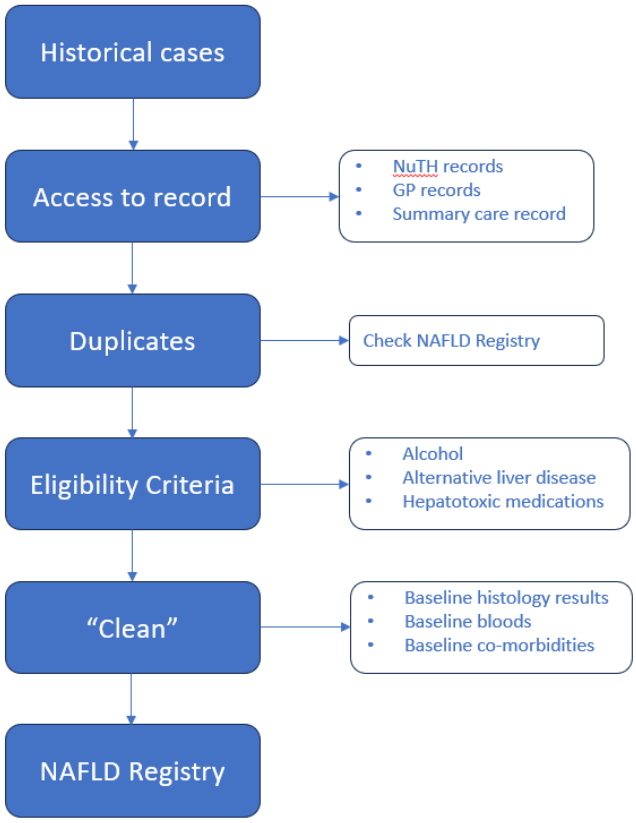
As the Newcastle NAFLD Registry was first created over twenty years ago, there are some data points that were not routinely collected for some years that now form the basis of a routine clinical review. This is in part due to the advancing knowledge of the importance of some clinical and laboratory features in NAFLD and the availability of newer clinical

investigations. Throughout the results chapters, any points of data collection that were not part of the first version of the registry will be highlighted to explain missing data numbers.

2.5.5. "Lost patients"

As described above, a number of patients eligible for inclusion in the study were identified from the historic clinical database of patients with NAFLD in the Newcastle Hospitals. This sub-cohort of patients' data would be added to the NAFLD Registry to allow analysis of the full Newcastle NAFLD cohort, but prior to their inclusion to the registry several steps were taken to ensure suitability and accurate data, as demonstrated by figure 2.1 below.

Figure 2.1. Methodology for inclusion of historical cases into Newcastle NAFLD cohort.



Firstly, searches for up-to-date clinical data were undertaken using hospital records, GP records and summary care record. Patients whose up-to-date information could not be obtained, in particular mortality status, were removed from the study. The historical cases were then checked against existing cases in the registry to ensure no duplication. Eligibility was then rechecked for these patients to ensure no new diagnosis of an alternative liver disease had been made and no use of prohibited medications such as Tamoxifen had occurred since their first clinical presentation. Records were also checked to ensure there was no suggestion of significant alcohol use for example in GP records of Audit-C tools or any

presentations to hospital with alcohol related injury or illness. Patients who did not meet the study inclusion criteria were then removed.

Once an appropriate list of eligible patients were identified, data was then added to the NAFLD Registry to allow for analysis of the complete cohort. As the data was being recorded every effort was made to ensure accuracy of results by reviewing the original clinical data and compiling information from hospital, GP and summary care records.

2.5.6 Data monitoring

The European NAFLD Registry data is monitored at various levels. Pre-existing patients within the registry have six-monthly central monitoring by the Registry Management Team, as well as local oversite by the chief investigator. Data queries raised by the central team are verified by the local hepatology research team. Data collected for the patients not previously consented also became subject to six-monthly monitoring upon it being entered into the database.

2.6 Statistical Methods

2.6.1 General approach

All statistical analyses were performed using SPSS version 26.0 & 28.0 (SPSS Inc. Chicago, IL). P-values were considered as statistically significant when $p<0.05$, unless otherwise stated, and exact p-values were used throughout. General approaches to statistical analysis conducted in this thesis are shown here and further, more detailed approaches will be shown in the subsequent chapters.

2.6.2 Missing data

The long-term outcomes in the Newcastle NAFLD study represent a “real world” cohort study. Every effort was made to ensure the data collected were as complete as possible, but as discussed above there were some data points that were not originally included in the registry and added at a later date, and for those patients no longer under follow-up it was not always possible to access full clinical details and up-to-date results. One notable example is in patients no longer receiving tertiary liver care, blood tests such as coagulation screen (PT) and some liver enzymes (AST, GGT) were not routinely measured and were therefore missing. Blood results were also not available for patients that had died during the follow-up. Given the missing results were predominantly from the group of patients no longer

under follow-up and not at random, the decision was made not to "impute" missing data results in an attempt to avoid introducing bias, and instead report the results as we found them, acknowledging the affect that missing results may have on any statistical analysis.

2.6.3 Normality

The normality of data collected in this study was predominantly assessed visually using histogram plots as well as exploring skewness, kurtosis, the mean (plus SD) and median (plus IQR). Where there was uncertainty of normality Kolmogorov-Smirnoff or Shapiro-Wilk tests were also performed to confirm, acknowledging the limitations of a large sample size on these tests. Variables found to be normally distributed were assessed using parametric tests, and non-normally distributed data were assessed using non-parametric tests.

2.6.4 Univariate analysis

Various different statistical tests were used for univariate analysis throughout this study. These tests were selected based on factors including normality assessments of the data being examined and the type of data for example continuous parametric data were explored using Student-t tests, non-parametric using Mann-Whitney U. Categorical variables with only 2 categories were analysed using Chi-Square or Fisher's Exact tests, and those with greater than 2 categories using Kruskal-Wallis. Two-sided tests were used unless otherwise specified and the statistical tests used are indicated throughout the results.

2.6.5 Multivariate Regression and Survival Analysis

Kaplan Meier log-rank tests and survival curves were undertaken to explore outcomes associated with all-cause mortality and liver related mortality.

To further explore factors which may predict mortality outcomes, Cox Regression proportional hazard modelling was utilised. Data found to be significant to the level of p=0.100 in univariate analysis were included in Cox regression. Prior to being included in a model each data point was assessed for "proportionality" by log-minus-log graph. Data found to be "non-proportional" in general were not included in any models, unless stated otherwise. A backwards stepwise regression model was used and then the results re-tested in a forced entry model to check significance. Variables were deemed not to be effective in controlling for the model once significant >0.100 and they were removed. Adjusted Hazard Ratios were displayed with 95% confidence intervals.

Binary logistic regression was used to explore outcomes not related to mortality such as progression of fibrosis. Again, a backwards stepwise approach was used, and Odds Ratios displayed with 95% CI.

Chapter 3: Cohort Baseline

3.1 Introduction

Despite NAFLD being the most common cause of liver disease worldwide, the natural history of the condition remains poorly understood. It is now well recognised that there is significant variability in how the condition progresses; the majority of patients will have stable non-progressive disease whilst 10-30% will develop a more progressive course.[9, 56] Gaining a better understanding of those that progress versus those that do not will be key to providing better long-term care for patients, with targeted screening for disease progression and long-term complications. Cohort studies with large patient numbers, diversity of disease severity and a long duration of follow-up are a vital part to bettering our understanding.

3.2 Aims

This chapter will:

1. Describe the baseline characteristics of the Newcastle Non-Alcoholic Fatty Liver Disease cohort.
2. Examine the co-morbidities present in the cohort.

3.3 Cohort Recruitment

3.3.1 Patient Selection

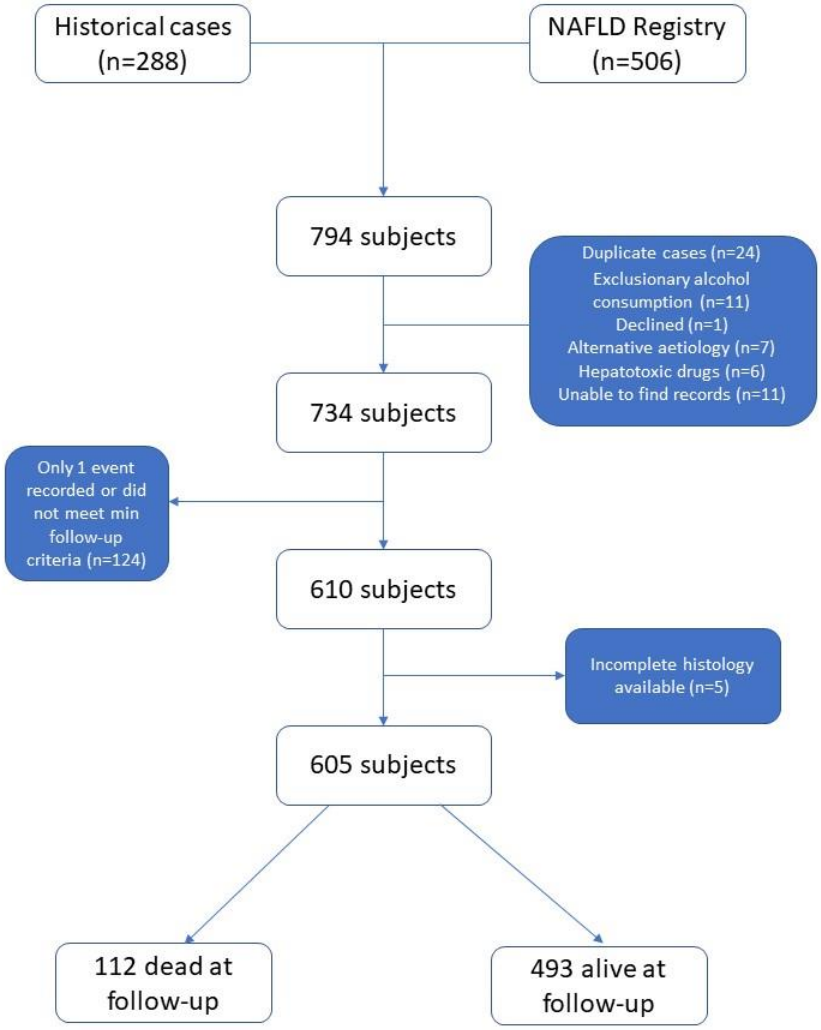
Subjects were identified from the Newcastle upon Tyne Hospitals clinical database of Non-Alcoholic Fatty Liver Disease.

A total of 794 patients were included in the database, each of these had been identified from specialist Hepatology clinics managed by experienced hepatologists from 1984 – 2019.

Five hundred and six of these patients remained under clinical care at Newcastle Hospitals and had given consent to take part in clinical research by way of the European NAFLD Registry, now supported through the LITMUS Consortium. The remaining 288 had been lost to follow-up for a variety of reasons including discharge from clinic, moved out of the region and some had died.

All 794 patients were screened for eligibility according to the criteria as listed in Chapter 2 and a total of 605 met the criteria for inclusion in this study as demonstrated by figure 3.1 below.

Figure 3.1. Review of eligibility criteria for potential participants in the Newcastle NAFLD cohort for patients identified in specialist Hepatology clinics from 1984-2019.



3.3.2 Recruitment timeline

The earliest patient identified in the NAFLD Register was recruited in July 1984. In order to allow a minimum of two clinical events with a minimum of twelve months follow-up recruitment ended March 2019.

3.3.3 Missing data

Missing data rates will be described throughout the results reporting. Given many of the patients included were recruited >20 years ago, there are some data points that were not routinely collected at the time of their inclusion, and the European NAFLD Registry has evolved substantially over the course of its existence. One pertinent example of missing data

for the cohort is smoking status, which made calculating certain cardiovascular risk scores such as the Framingham Risk Score for coronary artery disease[325] or the QRISK3[297] not possible.

As described in chapter 2, throughout the study missing data occurred most frequently in patients who were no longer under the care of the Newcastle Hospitals or had died. Every effort was taken to obtain data for these patients by accessing alternative hospital records or GP records where possible. Efforts were also made to "clean" any historic data, by checking records and correcting any errors, where found. Any data that were felt to be inaccurate and could not be verified were removed from the dataset. Given these missing data were reflective of a particular group of patients within the cohort there were concerns about introducing bias by imputation and therefore the decision was made not to impute missing data figures and keep the cohort as a "real world" example of data available for patients with NAFLD.

3.4 Patient Characteristics at Baseline

3.4.1 Baseline demographic, anthropometric and laboratory characteristics

Table 3.1 below displays the baseline characteristics of the Newcastle NAFLD cohort. "Normally" distributed results are displayed as mean ± standard deviation, non-parametric as median and range. Result have been rounded to decimal or integer numbers as would be used clinically, for example weight in kg is documented to 1 decimal place.

Clinical biochemical and haematology results were taken at the time of the original liver biopsy, or at recruitment if patient did not undergo a biopsy at this encounter.

Overall, 57% of the cohort was male and the median age at recruitment was 54 (18-83) years. In keeping with the local population of the North East of England the vast majority of patients (95%) were Caucasian. In total, 80.5% (473) of the cohort's BMI fell within the "obese" category (>30kg/m^2). Across the cohort the mean ALT, AST and GGT results were all elevated above the normal range, in keeping with the most recognised abnormal liver blood tests seen in patients with NAFLD.

Table 3.1. Baseline demographic data of the Newcastle NAFLD cohort. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	n	Result
Age (years)	605	54 (18-83)
Sex (male)	605	345 (57.0%)
BMI (kg/m^2)	587	34.0 (19.1-59.1)
Waist (cm)	464	112 ± 13
HbA1c (<42) [mmol/mol]	531	45 (20-117)
HDL (>1.0/1.2) [mmol/l]	501	1.10 (0.50-3.1)
LDL (<4) [mmol/l]	297	2.79 ± 0.07
Triglycerides (<1.7) [mmol/l]	521	2.10 (0.40-10.0)
ALT (0 – 40) [U/l]	590	61 (13-355)
AST (0 – 40) [U/l]	560	43 (13-251)
ALP (30-130) [IU/L]	590	92 (29-439)
GGT (0 – 70) [U/l]	533	82 (14-2062)
Albumin (35 – 50) [g/l]	590	45 ± 4
Platelets (150 – 450) [x10^9/l]	591	237 ± 75
PT (10 – 13) [seconds]	500	12 (9-19)
IgA (0.8 – 3.0) [g/l]	553	2.75 (0.70-20.90)

3.4.2 Baseline Disease Staging Scores

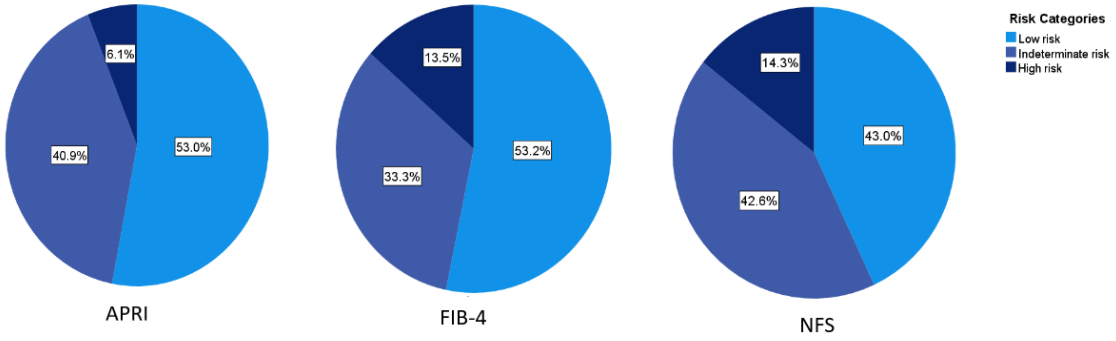
As per routine clinical care, non-invasive disease staging scores were calculated on patients at first presentation. As discussed in Chapter 1 there are many scores available, Newcastle Hospitals have traditionally used AST/ALT Ratio, APRI, FIB-4 Score and NAFLD Fibrosis Score which are all easily calculated with routinely available clinical data. The median results for each of the tools used are detailed below in Table 3.2.

Table 3.2. Results of baseline non-invasive staging tools calculated from routinely available clinical data in the Newcastle NAFLD cohort.

Non-invasive Tool	n	Result
AST/ALT Ratio	558	0.74 (0.22-2.91)
APRI	558	0.47 (0.08-3.97)
FIB-4 Score	556	1.26 (0.15-12.75)
NFS	488	-1.19 (-5.39-4.93)

Using the cut-off points derived from the original papers[157, 158, 162], three risk categories were created for APRI, FIB-4 score and NFS. These cut-offs were: 0.5 and 1.5 for APRI, 1.30 and 2.67 for the FIB-4 score, and -1.455 and 0.676 for the NFS. The figure below displays the distribution of patients in each category at the baseline visit.

Figure 3.2. Proportion of patients in each risk category by non-invasive tool at baseline. Cut offs used were APRI 0.5 and 1.5, FIB-4 1.30 and 2.67, and NFS -1.455 and 0.676.



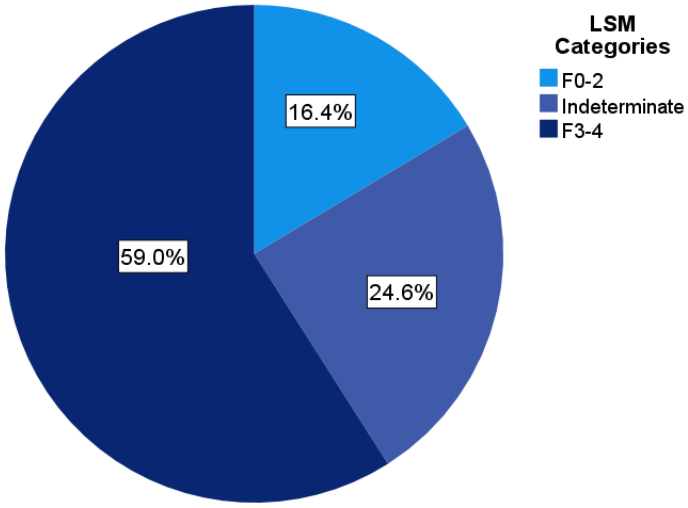
Clinical staging of disease can also be performed using Fibroscan™ as discussed in Chapter 1. This tool became widely clinically available in around 2014 and the Newcastle Hospitals have been able to perform these routinely since then. Consequently, a number of patients who were recruited and had a liver biopsy prior to this date do not have a "baseline" Fibroscan™ elastography result.

Overall, 10.1% (61) of the overall cohort had valid liver stiffness recorded at the time of baseline data entry and the median LSM was 12.5kPa. The full results are documented below in Table 3.3 and figure 3.2 below also shows the distribution of patients across the F0-2, indeterminate and F3-4 categories based upon the cut-offs <8.0kPa, 8.0-11.99kPa and >12.0kPa recommended by Boursier and colleagues.[192, 326]

Table 3.3. Baseline Fibroscan™ results

Fibroscan™	n	Result
Median Liver Stiffness (kPa)	61	12.5 (4.7-75.0)
Inter-Quartile Range (kPa)	61	1.8 (0.2-18.7)
IQR/Med %	61	16.3% ± 7.1%
CAP (dB/m)	43	345 (12-400)
Probe • M • XL	58	 20 (34.5%) 38 (65.5%)

Figure 3.3. Distribution of fibrosis stage based upon Fibroscan™ results at baseline. Cut-offs used were <8.0kpa, 8.0-11.6kPa and >12.0kPa as recommended by Boursier et al.



3.4.3 Baseline Histology

One of the biggest strengths of this cohort is the large proportion of patient who have undergone a diagnostic and staging liver biopsy. Of the 605 patients in the study, 576 (95%) have baseline histology results available from this initial inclusion biopsy. The remaining 29 patients were already clinically cirrhotic at the time of presentation and biopsy is not routine clinical practice to biopsy in this case given the significant risks associated with the procedure and the lack of impact this result would have on their clinical management and prognosis.

As discussed in Chapter 1, there are various histological scoring systems that have been developed to stage liver histology in NAFLD. The most widely accepted histological scoring

system is the NASH Clinical Research Network criteria (NASH CRN)[206], and this is the system the pathologists in Newcastle Hospitals used for the cohort.

Each of the 576 liver biopsies were read by one of two expert liver pathologists (Professor Alastair Burt, Dr Dina Tiniakos) which resulted in a high level of reliability of the biopsy reporting, protecting against inter-observer variability.

At baseline, 62% of the cohort had steatohepatitis (NASH) present on biopsy, defined as the presence of at least 1 point in each of steatosis, ballooning and inflammation as per the Bedossa *et al*[207] diagnostic algorithm. The remaining 215 participants (37.3%) have been defined as having "NAFL". As per the Bedossa *et al* algorithm, the presence or absence of fibrosis was not considered when defining NAFL vs NASH. Advanced fibrosis, defined as F3 or F4, was present in 33.2%. Table 3.4 below details the results of the baseline biopsies for the cohort.

In line with existing literature, "not significant" fibrosis was classified as those with F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Table 3.4. Baseline histology results of the Newcastle NAFLD cohort as defined by the NASH CRN criteria."Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Histological Measurement	n	Result
Steatosis Score • 0 • 1 • 2 • 3	576	 4 (0.7%) 174 (30.2%) 259 (45.0%) 139 (24.1%)
Ballooning Score • 0 • 1 • 2	576	 198 (34.4%) 253 (43.9%) 125 (21.7%)
Inflammation Score • 0 • 1 • 2 • 3	576	 139 (24.1%) 264 (45.8%) 162 (28.1%) 11 (1.9%)
Fibrosis Score • 0 • 1 • 2 • 3 • 4	576	 150 (26.0%) 111 (19.3%) 124 (21.5%) 118 (20.5%) 73 (12.7%)
Fibrosis category • No significant • Clinically significant • Advanced	576	 261 (45.3%) 315 (54.7%) 191 (33.2%)
NASH (present)	576	357 (62.0%)
NAS Score Categories • 1-2 • 3-4 • ≥5	576	 147 (22.5%) 204 (35.4%) 225 (39.1%)
SAF Activity Score	576	2 ± 1
Fibrosing steatohepatitis	576	262 (43.3%)

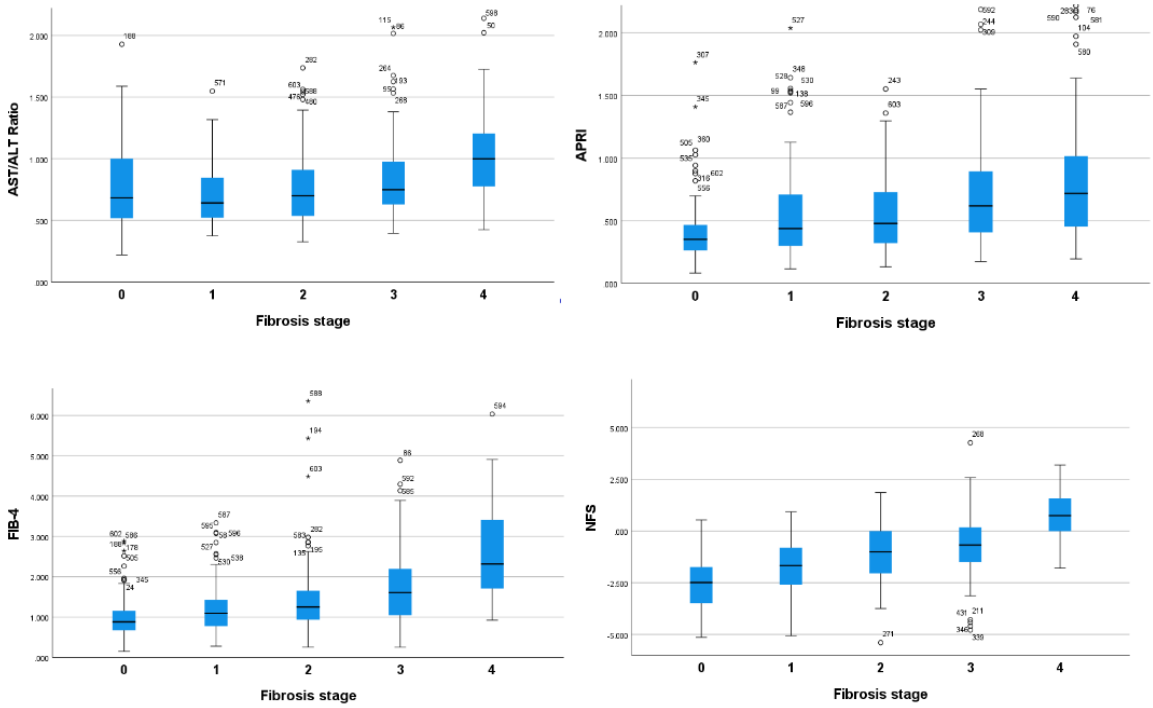
Of note there were 4 patients histologically defined as having <5% steatosis, all with F4. These patients were included in the study despite not meeting this inclusion criteria as a protocol deviation as they were all confirmed as having "features consistent with NAFLD" by the pathologist reviewing the biopsies.

Each of these 4 patients were rigorously reviewed for alternative diagnoses such as alcohol related liver disease or autoimmune liver disease. They were included in the cohort as these alternative aetiologies were excluded by other investigations and clinical picture.

3.5 Performance of non-invasive scores at baseline

The performance of the non-invasive tools in identifying histologically defined advanced fibrosis at baseline was explored. Figure 3.3 below shows the distribution of the AST/ALT ratio, APRI, FIB-4 and NFS scores corresponding to histologically confirmed fibrosis stage.

Figure 3.4. Boxplot illustrating distribution of the non-invasive scores (AAR, APRI, FIB-4 and NFS) related to histological fibrosis stage at baseline.

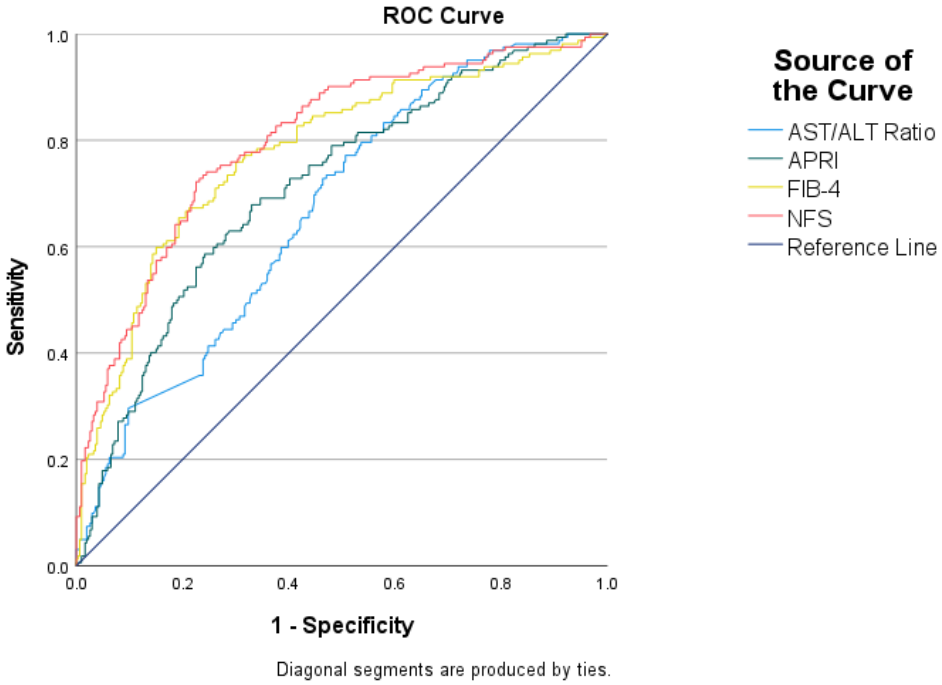


Area under receiver operating curves were then generated for the tools using F3-4 as the reference, as shown figure 3.4 below. The NFS tool performed the best with AUROC 0.801 (95% CI 0.76-0.84, p<0.001), followed by the FIB-4 score (AUROC 0.768, 95% CI 0.73-0.81, p<0.001). Table 3.5 below details the full performance of each tool and the sensitivity and specificity using the cut offs as detailed above.

Table 3.5. AUROC results for non-invasive tools demonstrating ability to predict F3-4 in the Newcastle NAFLD cohort.

Non-invasive tool	AUROC (95% CI)	p value	Cut-off	Sensitivity	Specificity
AST/ALT Ratio	0.675 (0.63-0.72)	<0.001	>1	36.1%	78.8%
APRI	0.699 (0.65-0.75)	<0.001	>1.5	9.8%	99.6%
FIB-4	0.768 (0.73-0.81)	<0.001	<1.3	73.2%	69.5%
			>2.67	25.1%	96.3%
NFS	0.801 (0.76-0.84)	<0.001	<-1.455	83.3%	59.8%
			>0.676	27.8%	96.7%

Figure 3.5. ROC curve of the non-invasive tools performance in predicting F3-4. This demonstrates NFS and FIB-4 performed best with AUROC 0.801 and 0.768 respectively.

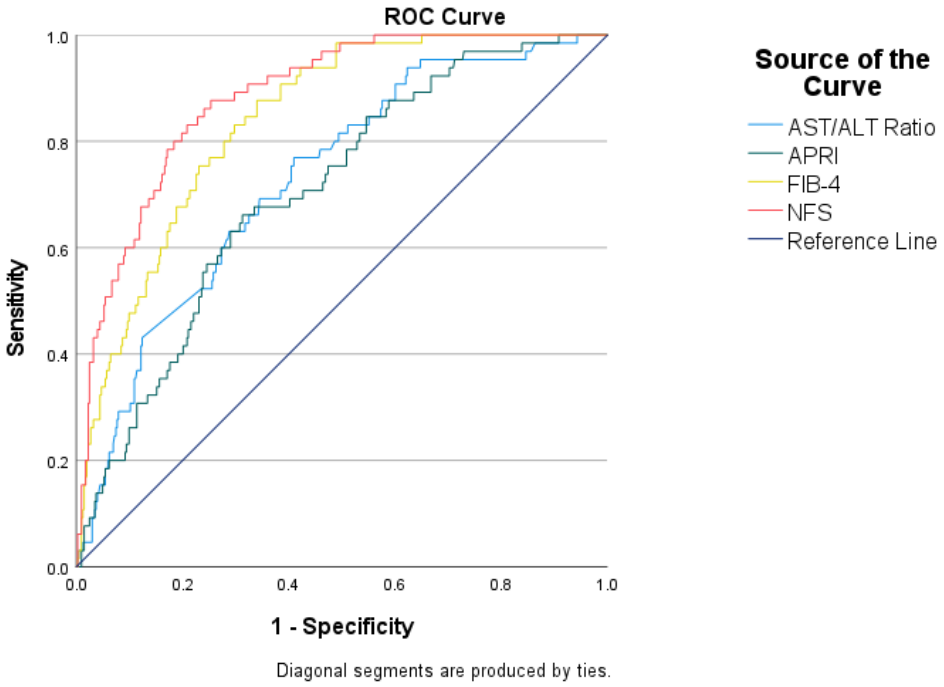


This process was repeated using F4 as the reference result and as can be seen from each of the scores in table 3.6 below there was an improvement in AUROC results and sensitivities across each score. In addition to established cut-offs previously used to diagnose cirrhosis, the use of recently proposed alternatives for each score by Brandman and colleagues[327] were also explored (AAR 0.88, APRI 0.54, FIB-4 1.67 and NFS 0.28). Figure 3.5 below displays the ROC curves for F4.

Table 3.6. AUROC results for non-invasive tools demonstrating ability to predict F4 in the Newcastle NAFLD cohort. Alternative cut-offs as proposed by Brandman et al were used (AAR 0.88, APRI 0.54, FIB-4 1.67 and NFS 0.28).

Non-invasive tool	AUROC (95% CI)	p value	Cut-off	Sensitivity	Specificity
AST/ALT Ratio	0.747 (0.69-0.81)	<0.001	0.88	65.2%	69.8%
			>1	55.1%	77.9%
APRI	0.710 (0.65-0.77)	<0.001	0.54	69.6%	62.1%
			>1.5	17.4%	96.1%
FIB-4	0.848 (0.81-0.89)	<0.001	1.67	76.8%	76.1%
			>2.67	42.0%	93.8%
NFS	0.883 (0.85-0.92)	<0.001	0.28	61.5%	88.8%
			>0.676	50.8%	94.5%

Figure 3.6. ROC curve of the non-invasive tools performance in predicting F4, using the new cut-offs as proposed by Brandman et al (AAR 0.88, APRI 0.54, FIB-4 1.67 and NFS 0.28). This again demonstrates NFS and FIB-4 performed best with AUROC 0.883 and 0.848 respectively.



3.6 Co-morbidities

As discussed in chapter one, NAFLD is associated with several other medical conditions. The incidence of some of the most observed co-morbidities in the cohort are described below.

3.6.1 Type 2 Diabetes Mellitus

The table below explores the differences in characteristics between the groups of patients with T2DM vs those without at the time of the baseline visit. Two hundred and eighty six (47.4%) of patients had a diagnosis of diabetes at their baseline record. Patients with a diagnosis of T2DM were older (median 58 years), had significantly higher BMI results (p<0.001) and a higher proportion were women (p=0.007). There were also notable differences in liver laboratory results including lower ALT (p<0.001), albumin (p<0.001) and platelet (p=0.002) results.

Table 3.7. Comparison of demographic characteristics between diabetes groups at baseline. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	T2DM (n=286)	No T2DM (n=317)	p value
Age	58 (18-83)	50 (18-82)	<0.001*
Sex (male)	51% (146)	62.1% (197)	0.007#
BMI (kg/m^2)	36.1 (22.7-59.1)	32.9 (19.1-56.5)	<0.001*
HbA1c$^+$	57.0 (29-117)	38.0 (20-102)	<0.001*
Fasting glucose#	7.9 (2.5-23.4)	5.3 (3.5-15.8)	<0.001*
ALT (0 – 40) [U/l]	53 (10-278)	65 (13-355)	<0.001*
AST (0 – 40) [U/l]	43 (13-230)	42 (13-251)	0.361*
GGT (0 – 70) [U/l]	92 (17-778)	74 (14-2062)	0.010*
Albumin (35 – 50) [g/l]	44 ± 4	45 ± 3	<0.001*
Platelets (150 – 450) [x10^9/l]	228 ± 80	247 ± 67	0.002~
PT (10 – 13) [seconds]	12 (9-19)	12 (10-17)	0.014*
IgA (0.8 – 3.0) [g/l]	3.12 (0.26-12.90)	2.50 (0.1-20.90)	<0.001*
APRI	0.50 (0.12 -3.18)	0.46 (0.08-2.82)	0.176*
FIB-4	1.53 (0.37-12.75)	1.09 (0.15-10.71)	<0.001*
NFS	-0.02 ± 1.51	-2.18 ± 1.37	<0.001~
Cirrhosis	91 (31.8%)	24 (7.5%)	<0.001#

*Mann Whitney-U test, # Chi-squared, ~Student t-test

$^+$HbA1c <42 mmol/mol = non-diabetes, >47mmol/mol diagnostic of diabetes. #

>7.0mmol/l = consistent with diabetes, 6.1-7.0mmo/l “impaired glucose handling”

The mean result of each non-invasive staging score was higher in the group of patients with diabetes (APRI p=0.176, FIB-4 score p<0.001 and NFS p<0.001) which may indicate more advanced disease in this group of patients.

Table 3.8 below displays the differences in baseline histology results between the groups. A significantly higher proportion of patients with T2DM had F4 on baseline histology than non-diabetic patients (p<0.001). There was also an associated increase in the number of patients with NASH (p<0.001) and the individual components of ballooning (p<0.001) and inflammation (p<0.001) histologically, although no significant difference in steatosis was noted (p=0.298).

Table 3.8. Comparison of baseline histology between diabetic groups as defined by the NASH CRN criteria. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	T2DM (n=263)	No T2DM (n=311)	p value
NASH	210 (79.8%)	164 (52.7%)	<0.001[#]
SAF Activity Score	3 ± 1	1 ± 1	<0.001[+]
NAS Score Categories • 1-2 • 3-4 • ≥5	 38 (14.4%) 90 (34.2%) 135 (51.3%)	 108 (34.7%) 113 (36.3%) 90 (28.9%)	<0.001[+]
Fibrosis • 0 • 1 • 2 • 3 • 4	 25 (9.5%) 32 (12.2%) 66 (25.1%) 81 (30.8%) 59 (22.4%)	 125 (40.2%) 78 (25.1%) 58 (18.6%) 37 (11.9%) 13 (4.2%)	<0.001[+]
Fibrosis categories • Insignificant • Significant • Advanced	 57 (19.9%) 206 (72.0%) 140 (49.0%)	 203 (64.0%) 108 (34.1%) 50 (15.8%)	<0.001[#] <0.001[#]
Fibrosing steatohepatitis	171 (59.8%)	91 (28.7%)	<0.001[#]
[+] Kruskal-Wallis test, [#] Chi-squared test.			

3.6.2 Hypertension

When collecting data for this NAFLD cohort, hypertension was defined as a recorded systolic BP >140mmHg, diastolic BP >80mmHg, an existing clinical diagnosis or the use of anti-hypertensive medication.

Table 3.9 below displays the baseline characteristics of each group at baseline. At the time of the initial visit 53% (276) of patients had a diagnosis of hypertension. The median age was higher in those with HTN (58 vs 48 years) and proportionally more women had hypertension (p<0.001). There was no significant difference in the mean blood pressure readings recorded at baseline clinical events between groups. Patients with HTN tended to have higher AST results (p=0.036) and lower albumin levels (p=0.009) than those without HTN at baseline.

Table 3.9. Comparison of demographic characteristics between HTN groups at baseline. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	HTN (n=276)	No HTN (n=245)	p value
Age	58 (18-83)	48 (18-78)	<0.001*
Sex (male)	135 (48.9%)	159 (64.9%)	<0.001#
BMI (kg/m^2)	35.8 ± 5.4	33.0 (19.1-59.1)	<0.001*
Systolic BP (mmHg)	136 ± 15	130 (104-182)	0.039*
Diastolic BP (mmHg)	78 ± 10	79 ± 11	0.696~
ALT (0 – 40) [U/l)	58 (10-355)	62 (13-308)	0.264*
AST (0 – 40) [U/l)	44 (13-230)	42 (13-251)	0.036*
GGT (0 – 70) [U/l)	87 (17-778)	74 (14-1141)	0.025*
Albumin (35 – 50) [g/l]	44 ± 3	45 (29-55)	0.009*
Platelets (150 – 450) [x10^9/l]	236 ± 75	241 ± 74	0.440~
PT (10 – 13) [seconds]	12 (9-19)	12 (9-17)	0.652*
IgA (0.8 – 3.0) [g/l]	2.94 (0.7-9.18)	2.56 (0.42-12.90)	0.019*
APRI	0.51 (0.12-3.18)	0.43 (0.08-2.82)	0.011*
FIB-4	1.56 (0.25-7.43)	1.05 (0.15-12.75)	<0.001*
NFS	-0.52 ± 1.58	-1.91 (-5.39-4.93)	<0.001*
Cirrhosis	67 (24.3%)	28 (11.4%)	<0.001#
*Mann-Whitney U test, #Chi-squared test, ~Student t test.			

Notably a large proportion of patients did not have their baseline blood pressure readings recorded as this was not included in the dataset for the NAFLD Registry for some time.

The mean results of the non-invasive scoring tools differed significantly across the two groups, those with HTN scored higher across each tool as shown in the table above.

Table 3.10 below shows the comparison of the HTN groups histology results at baseline. Almost three quarters (71.1%) of patients with HTN had NASH demonstrated on histology at baseline, compared to 54.0% of non-hypertensive patients ($p<0.001$) and there was a significantly higher proportion of advanced fibrosis and cirrhosis on biopsy in the hypertensive group ($p<0.001$). As with diabetes there was increased ballooning and inflammation seen on the biopsy in the group with HTN, but there was no difference to steatosis ($p<0.001$, $p<0.001$ and $p=0.816$ respectively.

Table 3.10. Comparison of baseline histology between HTN groups as defined by the NASH CRN criteria. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	HTN (n=263)	No HTN (n=239)	p value
NASH	187 (71.1%)	129 (54.0%)	<0.001[#]
SAF Activity Score	2 ± 1	2 ± 1	<0.001[+]
NAS Score Categories • 1-2 • 3-4 • ≥5	 49 (18.6%) 90 (34.2%) 124 (47.1%)	 77 (32.2%) 88 (36.8%) 74 (31.0%)	<0.001[+]
Fibrosis • 0 • 1 • 2 • 3 • 4	 38 (14.4%) 41 (15.6%) 58 (22.1%) 81 (30.8%) 45 (17.1%)	 90 (37.7%) 51 (21.3%) 51 (21.3%) 28 (11.7%) 10 (7.9%)	<0.001[+]
NASH	210 (79.8%)	164 (52.7%)	<0.001[#]
Fibrosis categories • Insignificant • Significant • Advanced	 79 (28.6%) 184 (66.7%) 126 (45.7%)	 141 (59.0%) 98 (41.0%) 47 (19.7%)	 <0.001[#] <0.001[#]
Fibrosing steatohepatitis	149 (54.0%)	87 (35.5%)	<0.001[#]
[+] Kruskal-Wallis test, [#] Chi-squared test.			

3.6.3 Dyslipidaemia

For the purposes of this cohort, the term "dyslipidaemia" is defined as raised triglycerides (>1.7mmol/l), reduced HDL (<1.0mmol/l for men, <1.3mmol/l for women) or the use of lipid lowering drugs such as statins.

At the time of the baseline visit the vast majority of patients had been diagnosed with dyslipidaemia; 81.8% (422) of the cohort. Of these patients, 56.9% were regularly taking lipid-lowering drugs such as statins. Patients with dyslipidaemia were younger (51 vs 55 years) and the diagnosis was more common in men (p=0.002) but there was no significant difference in BMI between the groups (p=0.460). Those with dyslipidaemia had higher total cholesterol levels (p=0.029), lower HDL (p<0.001) and higher triglycerides (p<0.001) in

keeping with the diagnostic criteria. This group also had higher mean albumin levels (p=0.002) but there was no other significant difference in liver laboratory results at baseline.

When comparing non-invasive staging scores at baseline, there was no significant difference between the two groups in any of the mean results (AAR p=0.058, APRI p=0.913, FIB-4 p=0.084, NFS p=0.236). There was also no significant difference in the distribution of patients across the risk categories (APRI p=0.913, FIB-4 p=0.084, NSF p=0.233), the proportions of each result can be seen in the figure below.

Two hundred and sixty three patients with dyslipidaemia (64.1%) had NASH histologically at baseline, and 15.9% (67) were cirrhotic. There was no significant difference in NAS categories (p=0.159), SAF activity (p=0.592) or fibrosis (p=0.716) between the groups

3.6.4 The Metabolic Syndrome

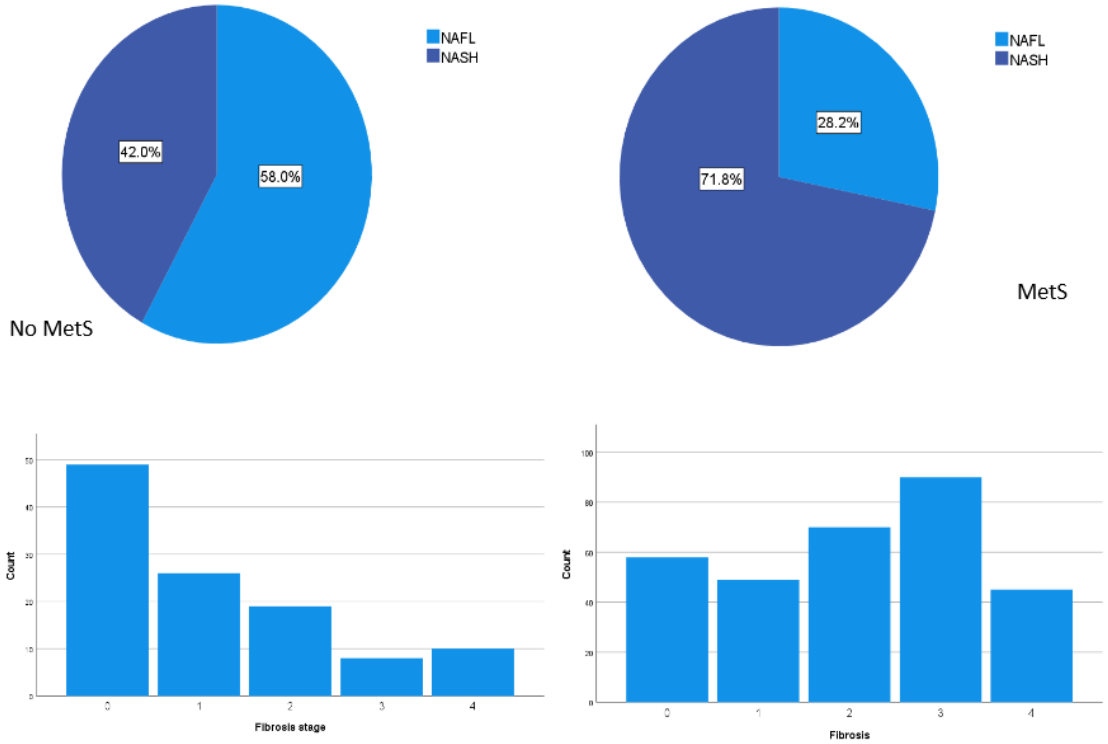
As detailed in chapter 1; the metabolic syndrome is defined as combination of any three of; central obesity, impaired fasting glucose, hypertriglyceridemia, low HDL and hypertension.[55] The table below outlines the comparison of demographic details between the group of patients with MetS vs those who did not meet the criteria. At the time of baseline visit, 74.1% (326) of the cohort had three or more features and were diagnosed as having the metabolic syndrome. These patients were older (56 vs 50 years) and had a higher mean BMI (p<0.001), however there was no significant difference in any of the liver related blood results. Importantly, there was a significantly higher proportion of those with MetS who had cirrhosis at baseline (21.2%) compared to those who did not meet the criteria (11.4%), p=0.025.

Table 3.11. Comparison of demographic characteristics between MetS groups at baseline. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	MetS (n=326)	No Mets (n=114)	p value
Age	56 (18-83)	50 (18-73)	<0.001*
Sex (male)	57.1% (186)	56.1% (64)	0.475#
BMI (kg/m^2)	35.4 ± 5.3	33.2 ± 5.8	<0.001~
No. of Mets features	4 (3-5)	1 (0-2)	<0.001*
ALT (0 – 40) [U/l)	60 (10-355)	69 (13-308)	0.223*
AST (0 – 40) [U/l)	43 (13-230)	44 (13-251)	0.827*
GGT (0 – 70) [U/l)	88 (17-778)	75 (14-2062)	0.083*
Albumin (35 – 50) [g/l]	45 (29-54)	45 ± 4	0.791*
Platelets (150 – 450) [x10^9/l]	240 ± 76	247 ± 73	0.396~
PT (10 – 13) [seconds]	12 (9-19)	12 (10-17)	0.177*
IgA (0.8 – 3.0) [g/l]	2.75 (0.7-12.9)	2.45 (0.72-7.84)	0.068*
APRI	0.47 (0.12-3.18)	0.46 (0.08-2.56)	0.457*
FIB-4	1.30 (0.25-12.75)	1.06 (0.15-10.71)	0.005*
NFS	-0.78 ± 1.77	-2.07 ± 1.69	<0.001~
Cirrhosis	69 (21.2%)	13 (11.4%)	0.025#
*Mann Whitney U test, #Chi Square test, ~ Student t test			

Differences in baseline histology between the MetS groups were explored. Given the presence of the MetS is associated with an increased risk of NASH and more advanced disease it is unsurprising to see that a much higher proportion of those who met the criteria for MetS had NASH (p<0.001), higher NAS scores (p<0.001), SAF activity scores (p<0.001) and more advanced fibrosis (p<0.001) than those who did not have MetS. Figure 3.6 below demonstrated the differences in NASH and fibrosis staging between the groups.

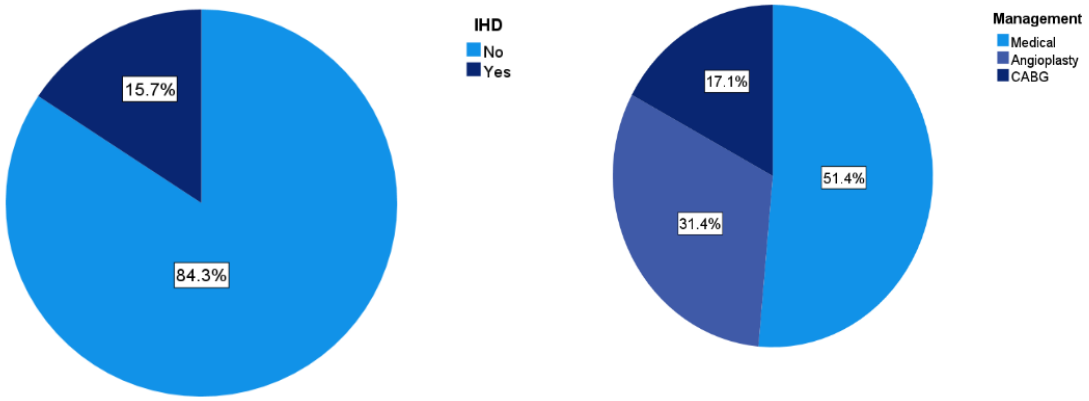
Figure 3.7. Differences in baseline histology, as defined by NASH CRN criteria, between MetS groups.



3.7 Clinical Outcomes

3.7.1 Ischaemic Heart Disease

Figure 3.8. Prevalence of IHD in the Newcastle NAFLD cohort at baseline and management methods.



As described in the co-morbidity section above, a significant proportion of the cohort were diagnosed with a number of risk factors for cardiovascular disease. At the time of inclusion into this study 15.7% (72) of patient had already experienced an ischaemic cardiac event; defined as a myocardial infarction or diagnosis of angina pectoris. Figure 3.7 above demonstrates the number of patients with IHD and the management of these patients, the

majority received medical management (51.4%) rather than angioplasty or coronary artery bypass graft surgery.

At baseline, patients with IHD were older (61 vs 52 years) and had significantly higher GGT results (p=0.026), lower albumin levels (p<0.001) and lower platelet count (p=0.027), but the remainder of the liver blood results were not significantly different. There was no significant difference in mean BMI. Table 3.12 below demonstrates the comparison of demographic between the two groups at baseline: those with IHD vs those without.

Table 3.12. Comparison of demographic characteristics between IHD groups at baseline. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	IHD (n=72)	No IHD (n=387)	p
Age	61 ± 9	52 (18-83)	<0.001*
Sex (male)	40 (55.6%)	221 (57.1%)	0.897#
BMI (kg/m^2)	35.3 ± 5.0	34.1 (19.1-59.1)	0.169*
ALT (0 – 40) [U/l)	58 (13-278)	60 (10-319)	0.245*
AST (0 – 40) [U/l)	43 (19-225)	42 (13-251)	0.671*
GGT (0 – 70) [U/l)	105 (19-1141)	81 (14-778)	0.026*
Albumin (35 – 50) [g/l]	43 ± 5	45 ± 3	<0.001+
Platelets (150 – 450) [x10^9/l]	221 ± 83	242 ± 72	0.027+
PT (10 – 13) [seconds]	12 (10-16)	12 (9-19)	0.989*
IgA (0.8 – 3.0) [g/l]	3.23 (0.7-9.18)	2.65 (0.42-12.90)	0.005*
APRI	0.51 (0.13-2.80)	0.46 (0.08-3.18)	0.061*
FIB-4	1.85 (0.43-12.75)	1.21 (0.15-10.71)	<0.001
NFS	-0.24 ± 1.86	-1.28 ± 1.74	<0.001+
*Mann Whitney U test, # Chi-square, +Student T test			

Patients with IHD also had a higher incidence of co-morbid conditions. Over sixty percent (63%) had Diabetes (p=0.007), 80% HTN (p<0.001), 73.5% Dyslipidaemia (p<0.001), and 89% met the criteria for the diagnosis of MetS (p=0.002) at baseline.

Whilst the non-invasive scoring tools were not developed to prognosticate for cardiovascular disease it is interesting to note that there was a significant difference in the FIB-4 and NFS

scores between the groups (p<0.001 for both), with a higher proportion of patients with IHD in the "high risk" category (p<0.001 for both). This appears largely due to the age difference between the groups as when controlling for age neither the FIB-4 or NFS remained significant (p=0.330 and p=0.486 respectively).

At the time of baseline liver biopsy, 73.8% of patients with IHD had NASH (p=0.038) and there was a trend to more advanced fibrosis stage (p=0.016) with significantly more cirrhotic patients (p=0.004) compared to those without IHD. However, after controlling for age, sex and BMI there was no longer a significant association between IHD and NASH (p=0.377), advanced fibrosis (p=0.991) or cirrhosis (p=0.165), as can be seen in table 3.13 below.

Table 3.13. Odds ratios demonstrating association of IHD with histology at baseline. At baseline there were significant differences in the presence of NASH (p=0.038), advanced fibrosis (p=0.016) and cirrhosis (p=0.004) between those with IHD compared to those without. These factors were controlled for age, sex and BMI and were no longer significant as can be seen in the table below.

Variable	IHD	OR (95% CI)	aOR (95% CI)*	p[#]
NASH	48 (73.8%)	1.93 (1.07-3.47)	1.33 (0.70-2.52)	0.377
Advanced Fibrosis	36 (50%)	1.61 (0.94-2.75)	0.91 (0.50-1.63)	0.740
Cirrhosis[+]	24 (25.8%)	2.30 (1.32-4.01)	1.24 (0.66-2.33)	0.503
*Corrected for Age, sex and BMI. [#] Binary regression. [+]Including clinical cirrhosis.				

3.7.2. Malignancy

As discussed in chapter one, the incidence of malignancy in NAFLD populations worldwide has been reported to be proportionally higher than that of the general population. The occurrence of malignancy within the Newcastle NAFLD cohort was therefore an endpoint of interest and data were recorded to explore this.

At the time of the baseline entry 6.5% (34) of the cohort had a current or prior cancer diagnosis. The most common malignancies were renal cell carcinoma (20.6%), breast cancer (14.7%) and skin cancers (14.7%), including SCC, BCC, and melanoma. Of interest in the Newcastle NAFLD group there was only 1 diagnosis of colorectal cancer which has been described as the most common malignancy in those with NAFLD and MetS.[328-330]

Patients with malignancy were older (63 vs 53 years) and there was a trend to more advanced liver disease at baseline, cirrhosis p=0.004. Table 3.14 below displays the characteristics of the malignancy groups at baseline.

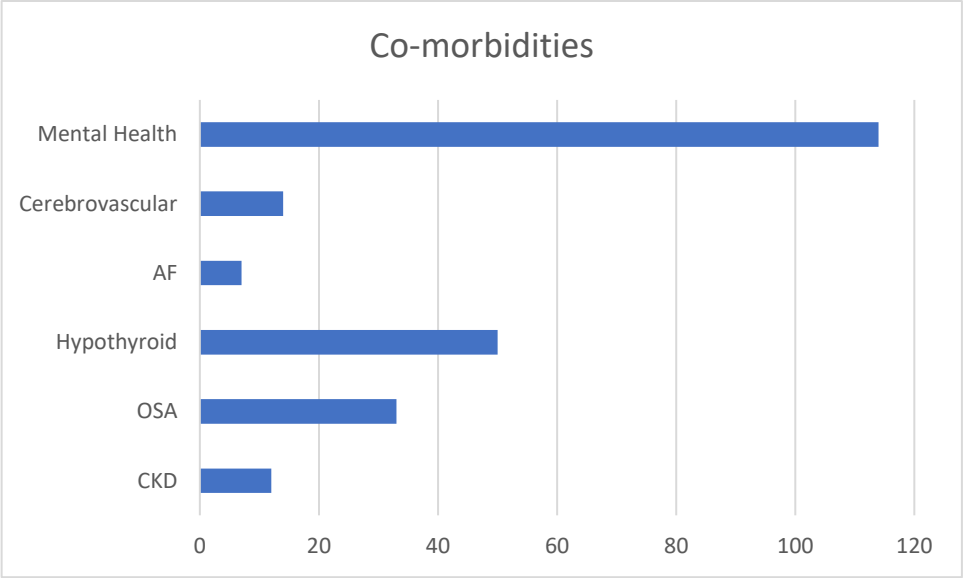
Table 3.14. Comparison of demographic characteristics between malignancy groups at baseline. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Malignancy (n=34)	No Malignancy (n=486)	p
Age	63 ± 9	53 (18-83)	<0.001*
Sex (male)	15 (44.1%)	279 (57.4%)	0.153#
BMI (kg/m^2)	35.9 ± 5.8	34.2 (19.1-59.1)	0.239*
ALT (0 – 40) [U/l)	52 (14-151)	62 (10-355)	0.012*
AST (0 – 40) [U/l)	44 ± 21	43 (13-251)	0.258*
GGT (0 – 70) [U/l)	57 (18-485)	84 (14-1141)	0.013*
Albumin (35 – 50) [g/l]	45 (32-50)	45 ± 4	0.031*
Platelets (150 – 450) [x10^9/l]	194 ± 58	238 (60-498)	<0.001*
PT (10 – 13) [seconds]	12 ± 1	12 (9-19)	0.862*
IgA (0.8 – 3.0) [g/l]	3.26 (0.47-9.83)	2.71 (0.7-12.90)	0.269*
APRI	0.54 (0.14-1.64)	0.68 (0.08-3.18)	0.462*
FIB-4	1.71 (0.86-4.87)	1.23 (0.15-10.71)	<0.001*
NFS	0.19 ± 1.42	-1.21 ± 1.77	<0.001+
NASH	21 (70.0%)	298 (63.3%)	0.559#
Cirrhosis	13 (38.2%)	84 (17.3%)	0.004#
*Mann-Whitney U test, +Student t-test, #Chi-square, ~Fisher's Exact			

3.7.3. Other co-morbidities

The primary co-morbidities of interest for the study are detailed above. There were however several other co-morbid conditions which were included in data collection for the cohort. Figure 3.8 below demonstrates the prevalence of these co-morbidities.

Figure 3.9. Prevalence of "other" co-morbidities at baseline.



AF= atrial fibrillation, OSA= obstructive sleep apnoea, CKD= chronic kidney disease.

The most prevalent "other" co-morbidity in the cohort was a mental health diagnosis. One hundred and fourteen patients (25.2% of the cohort) had been diagnosed with Depression or Anxiety by the time of inclusion in the study. Hypothyroidism was the second most common other co-morbidity, present in 11.2% (50). Obstructive Sleep Apnoea (OSA) was present in 6.6% (33) and 62.5% of these patients using continuous positive airway pressure therapy (CPAP) at night as management for this. Seven (1.5%) patients had a diagnosis of Atrial Fibrillation (AF) at the time of the baseline visit and 3.0% (14) had been diagnosed with Cerebrovascular disease, defined as stroke or transient ischaemic attack (TIA). Chronic kidney disease (CKD) was present in 2.7% (12) of the cohort.

3.7.4 Bariatric Surgical Procedure

Bariatric procedures remain one of the most consistently effective management methods for obesity and related complications such as NAFLD. It is therefore interesting that only a very small proportion of the cohort had undergone any bariatric procedure; 0.6% (3) at baseline.

3.8 Cirrhosis at Baseline

At the time of baseline data collection 19.2% (116) of the cohort had a diagnosis of cirrhosis. Three quarters (87) of these were histologically diagnosed with liver biopsy whereas 9.5% (11) were diagnosed by imaging including ultrasound, CT scan or Fibroscan™, and the

remaining 15.6% (18) were clinically diagnosed using a combination of signs on physical examination, blood results and non-invasive staging scores.

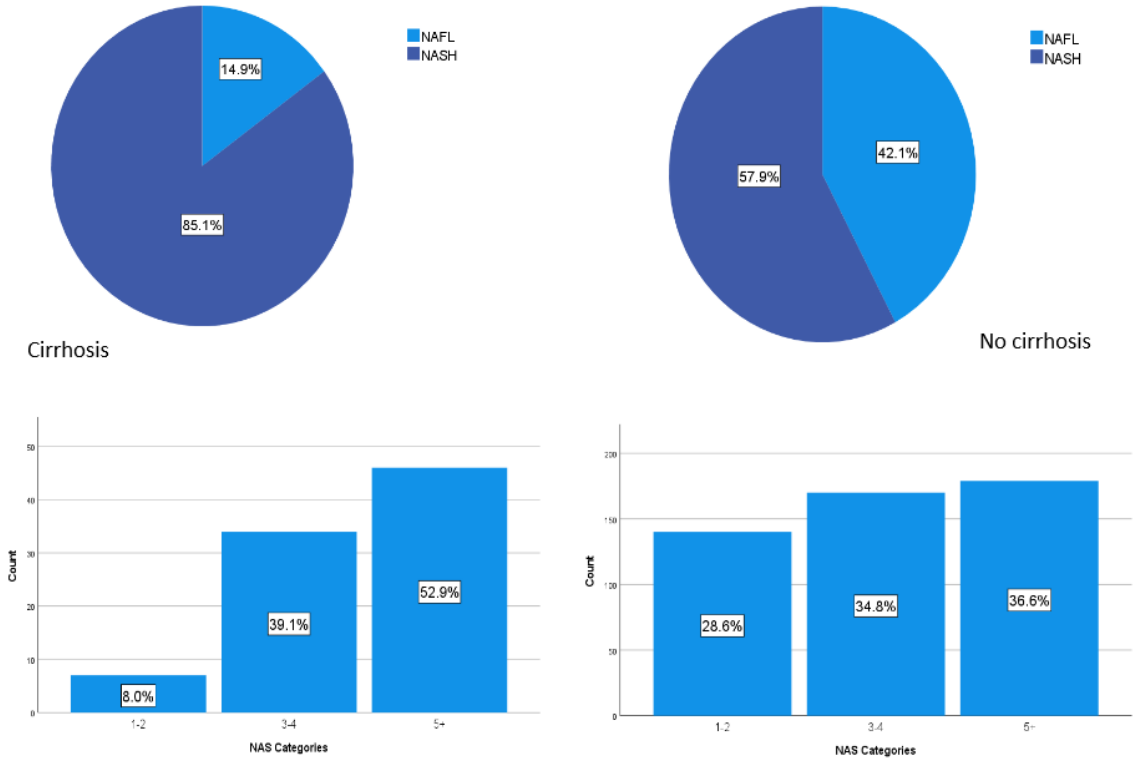
Table 3.15 details the characteristics from the compared groups at baseline. Patients with cirrhosis were significantly older (p<0.001) and of note a significantly higher proportion of women had cirrhosis than men (p=0.001). There were also notable differences in liver laboratory results with significantly lower ALT p<0.001, albumin p<0.001, and platelets p<0.001, and, as expected, significantly higher non-invasive scoring results (p<0.001) in those with cirrhosis.

Table 3.15. Comparison of demographic characteristics between cirrhosis groups at baseline. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Cirrhosis (n=116)	No Cirrhosis (n=489)	p value
Age	60 (18-83)	52 (18-76)	<0.001*
Sex (male)	40.5% (47)	60.9% (298)	<0.001#
BMI (kg/m^2)	36.0 ± 6.0	33.7 (19.1-59.1)	0.007*
ALT (0 – 40) [U/l)	46 (13-221)	63 (10-355)	<0.001*
AST (0 – 40) [U/l)	48 (18-218)	42 (14-1141)	0.070*
GGT (0 – 70) [U/l)	131 (23-2062)	74 (14-1141)	<0.001*
Albumin (35 – 50) [g/l]	43 (27-51)	45 ± 3	<0.001*
Platelets (150 – 450) [x10^9/l]	172 ± 69	242 (86-498)	<0.001*
PT (10 – 13) [seconds]	12 (10-19)	12 ± 1	<0.001*
IgA	4.03 (0.1-12.90)	2.53 (0.26-20.90)	<0.001*
APRI	0.73 (0.20-3.97)	0.43 (0.08-3.18)	<0.001*
FIB-4 score	2.64 (0.93-12.75)	1.10 (0.15-7.43)	<0.001*
NFS	0.84 ± 1.42	-1.62 ± 1.54	<0.001+
*Mann Whitney U test, # Chi-squared test, +Student t-test			

As would be expected, a significantly higher proportion of those with cirrhosis at baseline also met the criteria for NASH on baseline biopsy (85.1% vs 57.9%, p<0.001) and these patients also had higher NAS scores (p<0.001) and SAF activity scores (p<0.001). Figure 3.9 below displays the difference in histology at baseline between these groups.

Figure 3.10. Comparison of baseline histology, as defined by NASH CRN criteria, between cirrhosis groups.



When comparing the presence of co-morbidities between the groups there was a significantly higher proportion of patients with cirrhosis who had T2DM (p<0.001), HTN (p<0.001), MetS (p=0.025) and IHD (p=0.004). However, after correcting for age and sex the only remaining relevant association was the presence of T2DM. The odds ratios and adjusted ORs for these co-morbidities are shown in table 3.16 below.

Table 3.16. Odds ratios demonstrating associations with co-morbidities and the presence of cirrhosis at baseline. There were a significantly higher proportion of patients with cirrhosis who had T2DM (p<0.001), HTN (p<0.001), MetS (p=0.025) and IHD (p=0.004) at baseline. However, after correcting for age and sex, T2DM was the only co-morbidity that remained significantly associated with cirrhosis as can be seen in the table below.

	Incidence	Odds Ratio (95% CI)	Adjusted OR (95% CI)*	p value#
T2DM	91 (79.1%)	5.70 (3.51-9.25)	3.74 (2.18-6.40)	<0.001
HTN	67 (70.5%)	2.48 (1.53-4.02)	1.17 (0.67-2.03)	0.590
Dyslipidaemia	65 (70.7%)	2.74 (1.60-4.46)	1.71 (0.99-2.95)	0.053
MetS	69 (84.1%)	2.09 (1.11-3.94)	1.39 (0.69-2.81)	0.359
IHD	24 (25.8%)	2.30 (1.32-4.01)	1.31 (0.71-2.43)	0.387
Malignancy	13 (13.4%)	2.96 (1.43-6.15)	1.34 (0.60-3.01)	0.476
*Adjusted for age, sex and BMI. #Binary regression.				

3.9 Chapter summary.

Six hundred and five individuals with a diagnosis of NAFLD met the eligibility criteria for inclusion in this study assessing the long-term outcomes of NAFLD. Overall, 95.2% (576) had baseline histology at the time of inclusion into the study and 19.2% (116) were cirrhotic. In keeping with previous studies, the FIB-4 and NFS scores identified advanced fibrosis and cirrhosis with reasonable accuracy. The cohort had significant comorbidity at baseline including a high prevalence of T2DM (47.4%), HTN (53.0%), MetS (74.1%) and IHD (15.7%). The presence of baseline diabetes was associated with an increased risk of cirrhosis, even after controlling for age, sex, and BMI, aOR 3.74 (95% CI 2.18-6.40).

3.10 Discussion

This chapter describes the baseline clinical characteristics of the Newcastle NAFLD cohort, which is one of the largest and longest established single centre NAFLD cohorts in the world.

When comparing the results of this cohort to the existing NAFLD literature or the general population it is important to recognise that this cohort has been collected from a tertiary liver centre, which automatically biases the patient selection towards more advanced disease. This will be reflected in multiple aspects of the cohort, not only relating to the liver results, but also to the concurrence of other medical issues. However, given the primary aim of this study is to investigate the long-term outcomes of patients with NAFLD this will not

affect the accuracy of these outcomes but may increase the proportion of outcomes experienced compared to the general population.

Overall, the cohort demographics at the time of baseline are in keeping with what has been broadly described in other studies; patients were “middle aged”, obese according to BMI and the population was predominately male. Baseline liver blood results were also reflective of the overall literature with raised ALT and GGT in particular being well reported [56, 93].

When comparing to recent literature, the performance of the non-invasive tools at identifying advanced fibrosis in the Newcastle NAFLD cohort was similar to what was shown in the recent STELLAR trials.[171] This study showed the FIB-4 score performed best with an AUROC 0.78 and NFS AUROC 0.74, compared to FIB-4 AUROC 0.768 and NFS AUROC 0.801 in the Newcastle cohort. Using the higher cut-offs (FIB-4 = ≥2.67 and NFS = ≥0.676) similar sensitivity and specificity results were also seen.

The baseline histology of the cohort reflects more advanced disease seen as a result of the cohort being derived from a tertiary centre hospital. In the early years of the recruitment to the Newcastle NAFLD cohort clinicians were highly selective of the patients who required care in a secondary/tertiary setting based upon their clinical judgement and factors such as co-morbidities etc, and in 2009 this process was strengthened by the implementation of non-invasive score selection of patients that required specialist management. This is demonstrated by the high proportion of patients with NASH within the cohort (62% at baseline). The prevalence of NASH in the general population is difficult to estimate, but several studies have suggested it ranges from 1.5% to 12%. [2, 28, 50] Within the NAFLD population it is felt to be present in 10-40%, but within the highly selective group of patients that take part in clinical trials or cohort studies, which informs a large amount of the knowledge of the condition, this number can be as high as 50-70% of patients [33, 50, 93, 98, 252].

This issue of high-level selectivity of cases can also be demonstrated in the proportion of the Newcastle cohort who have advanced fibrosis (F3 or F4). It is widely reported that 25-40% of patients with NAFLD in a secondary care setting will develop progressive liver fibrosis, ultimately resulting in cirrhosis in 10-20% [33, 95, 97, 99, 212, 331]. The prevalence within the Newcastle cohort (33.2%) is therefore in keeping with what has previously been demonstrated within the literature. However, the question of general population prevalence remains as there are very few studies which have sought to explore this as liver biopsy is not

always the most accessible investigation to confirm fibrosis stage. In a study of the general population of Hong Kong, V. Wong *et al*[332] used elastography to assess for advanced fibrosis (classified as >9.6kPa) in 759 randomly selected subjects. In that study, 217 (28.6%) people were shown to have steatosis >5% and 8 (3.7%) were found to have liver stiffness >9.6kPa. In a more multi-national systematic review by Harris *et al*[333] demonstrated the prevalence of liver fibrosis, by elastography or biomarkers including the FibroTest, in a general population to be between 0.7-25.7%. The significant variation in prevalence in this review is likely due to the use of 11 different non-invasive tests to stratify fibrosis and the different countries included which ranged from The Gambia to Hong Kong and the U.K. In a European cohort Gines *et al*[334] found the prevalence of fibrosis to be 0.7-7.5% which increased to 18-27% in cohorts which were more selective for risk factors such as T2DM, which is in keeping with the Newcastle cohort.

As discussed in chapter one, there are well documented bidirectional relationships recognised between NAFLD and other co-morbidities including Obesity, T2DM, Hypertension, Dyslipidaemia, and Ischaemic Heart Disease.

The global prevalence of NAFLD in patients with T2DM has been reported as high as 55.5%. Conversely, the prevalence of T2DM in patients with NAFLD has been shown in several cohort studies to vary between 8.5-40% [95, 98, 99, 255, 256]. However in a small cohort study with a prolonged follow-up period, this reached as high as 75% after a mean follow-up of 16.5 years [32]. With almost 50% of the Newcastle cohort having a diagnosis of T2DM at the baseline visit, the prevalence of T2DM is on the higher side of what has been previously reported in the literature. Many of these studies are historical and with rising rates of obesity in the United Kingdom, the general population prevalence of T2DM is increasing and therefore the prevalence of diabetes within the Newcastle NAFLD cohort is likely a reflection of a more current population study. This high prevalence also likely reflects early recognition of disease progression in patients with T2DM by clinicians in the Newcastle centre who were more likely to follow-up and biopsy patients with diabetes as was seen in other work from the hospital.[33]

This high prevalence of diabetes within the cohort is likely a contributing factor to the proportion of patients with advanced disease at baseline. A recent meta-analysis of 12 observational studies showed a two-fold increased risk of severe liver disease events and other studies have shown diabetes to be the strongest predictor for progressive disease [20,

[30]. Of the patients in the Newcastle cohort who had cirrhosis at baseline 79% had T2DM and after controlling for age, BMI and sex this remained a significant risk factor (aOR 3.74, 95% CI 2.18-6.40, p<0.001).

Hypertension is also very commonly reported in patients with NAFLD. Several natural history cohort studies have reported a baseline prevalence of 30-60% [95, 97-99, 255], which increases to 70-90% after a follow-up period [32, 93]. This is in keeping with the findings from the Newcastle cohort. Of particular note, the Newcastle cohort demonstrate a proportional increase in cirrhosis in those with hypertension compared to those without; p<0.001 (OR 2.48; 95% CI 1.53 – 4.02) at baseline. Singh *et al* [45] also reported in a systematic review, of 11 paired biopsy studies including 411 patients, that hypertension was associated with an increased risk of the development of progressive fibrosis (OR 1.94; 95% CI 1.00-3.74) although this has not been consistently seen in other studies.[20]

In summary this chapter reported the baseline characteristics of patients in the Newcastle NAFLD cohort. The proportion of advanced disease is significantly higher than reported in the general population but is in line with other highly selective studies and reflective of a tertiary centre population. The non-invasive fibrosis scores FIB-4 and NFS have been demonstrated to perform well at identifying advanced fibrosis in the diagnostic context of use. High prevalence of co-morbidities including HTN, IHD and the MetS has been identified but most importantly the presence of T2DM has been shown to be a significant risk factor for advanced disease. The relationship between co-morbidities and disease progression within this cohort will be further explored in the next chapter which will examine the follow-up characteristics of the Newcastle NAFLD cohort.

Chapter 4: Cohort Follow-up

4.1 Introduction

Hepatic steatosis was first described in the English language medical literature by Addison in 1836[335], and in 1980 Ludwig *et al*[202] recognised NAFLD as a form of chronic liver disease independent from alcohol related steatosis. For many years NAFLD was thought to be a benign, indolent disease however, more recently there has been much research to show the contrary: NASH occurs in 20-40% of patients with NAFLD and this in turn can lead to the development of fibrosis and cirrhosis in 10-20%.[56, 336] There has also been evidence to show significant associations to other co-morbidities and clinical outcomes of interest such as T2DM and IHD, which has prompted further work examining the long-term outcomes of NAFLD.[337] There have been many multi-centre studies and population studies that have attempted to address this, but the literature lacks detailed long-term outcome data from highly characterised single centre cohorts with a large number of histologically characterised participants.

4.2 Aims

This chapter will:

1. Describe the characteristics of the Newcastle Non-Alcoholic Fatty Liver Disease cohort at the last clinical follow-up event or upon death.
2. Examine the co-morbidities present at the time of the last follow-up event and the changes that have occurred over the follow-up period.
3. Define the cause of death in the patients who died.
4. Describe the characteristics of patients that died of all causes.
5. Explore any factors associated with all-cause mortality and any features which may predict mortality.
6. Examine the non-invasive scores' prognostic ability for all-cause mortality.

4.3 Patient Characteristics at Follow-up

4.3.1 Follow-up demographics and anthropometric characteristics

The mean duration of follow-up of all patients in the Newcastle NAFLD cohort was 12years (1.0-35.4) and at this time 112 patients had died (18.5%), 493 (81.5%) were still alive. The median age at the final follow-up event, including death events, was 65 years. The median BMI for the cohort at follow-up remained within the "obese" category (33.6kg/m^2) as it was at the baseline visit. Table 4.1 below details the follow-up characteristics of all patients, including those that had died where results from the time of death were available.

As in chapter 3, parametric results in this chapter are displayed as mean ± standard deviation and non-parametric as median (IQR). Result have been rounded to decimal or integer numbers as would be used clinically, for example weight in kg is documented to 1 decimal place.

Table 4.1. Follow-up demographic data of the Newcastle NAFLD cohort. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range. Results were rounded to decimal or integer numbers as would be used clinically.

Variable	n	Result
Length of follow-up (months)	605	144 (12-425)
Age (years)	605	65 (29-97)
BMI (kg/m^2)	243	33.6 (23.5-52.5)
Waist (cm)	224	112 (53-149)
ALT (0 – 40) [U/l)	466	32 (7-314)
AST (0 – 40) [U/l)	288	30 (12-680)
GGT (0 – 70) [U/l)	209	61 (10-2002)
Albumin (35 – 50) [g/l]	466	45 (21-74)
Platelets (150 – 450) [x10^9/l]	446	219 (24-648)
PT (10 – 13) [seconds]	250	12 (9-49)
IgA (0.8 – 3.0) [g/l]	71	2.55 (0.62-7.46)

In comparison to the baseline data there are significantly more missing data-points at follow-up. The notable variation in the availability of the above clinical blood tests represents the difference in those who remained under the care of a Hepatology clinical team vs those who have been lost to follow-up or discharged. Laboratory results such as AST, GGT and PT are

only routinely performed by specialist liver clinics. Whereas ALT, Albumin and Platelets are considered to be generally routine blood tests. This therefore explains the significant increase in availability of ALT, Albumin and Platelet results at follow-up. In those who had died these details were also not routinely recorded.

4.3.2. Follow-up Disease Staging Scores

Table 4.2 below documents the results of the repeat prognostic assessments for the cohort at the time of their last clinical event or follow-up. The non-invasive scores used at follow-up were the AST/ALT Ratio, APRI, FIB-4 Score and NAFLD Fibrosis Score. These were the same as at the baseline and were chosen because they are easily calculated using routinely available clinical data. The mean results of both the FIB-4 and NFS score for the cohort at follow-up fall into the "indeterminate" range, whereas the median APRI score falls into the "low risk" category.

Table 4.2. Results of follow-up non-invasive staging tools calculated from routinely available clinical data in the Newcastle NAFLD cohort.

Prognostic Tool	Result n=288
AST/ALT Ratio	0.90 (0.12-0.26)
APRI	0.35 (0.06-4.99)
FIB-4 Score	1.46 (0.34-8.37)
NFS	-0.31 ± 1.56
Child's Pugh Score	5 ± 2

As each of these non-invasive scoring systems require the use of AST in the calculation, the number of available repeat scores is reflective of the above discussion point regarding routinely available clinical blood tests. The NAFLD Fibrosis Score (NFS) as discussed in Chapter 1, also requires the use of BMI and the presence or absence of diabetes for the calculation. In those patients who had become lost to follow-up this information was less readily available, and again this is demonstrated in the larger numbers of missing results at follow-up.

It is also worth noting that whilst the mean age of the cohort at the time of the final clinical event is 64yrs old, 51.4% were 65 years and over. As discussed in chapter 1, this is known to affect the accuracy of each of these tools and the British Society for Gastroenterology have

released clinical guidelines recommending the use of different cut off thresholds for patients ≥65 years when interpreting the FIB-4 Score and the NFS [108, 111]. Table 4.3 below demonstrates the mean results for these tools categorised into appropriate age groups and clearly demonstrates that those >65yrs have higher mean scores which is not necessarily reflective of more advanced disease.

Table 4.3. Age categorised non-invasive staging tool results, grouping patients by < and > 65years old as recommended by the BSG when interpreting the FIB-4 and NFS as the ALT is known to be affected by age and can influence results in those over 65yrs old.

Prognostic Tool	<65yrs old n=166	≥65 yrs old n=118
FIB-4 Score	1.19 (0.34-8.30)	2.07 (0.64-8.37)
NFS	-0.63 ± 1.55	0.17 ± 1.47

Using the age corrected cut-offs (<0.12, 0.12-0.676, >0.646 for NFS and <2.0, 2.0-2.67, >2.67 for FIB-4 in those over 65), as described above, the results of the scores were categorised and the proportion of patients with low, indeterminate, and high risk at the follow-up visit are shown in the figures below. These figures also demonstrate the movement of individuals across the risk categories with time. Interestingly there were very low numbers of individuals that moved from the "low" to "high" risk category with time.

Figure 4.1. Sankey diagram demonstrating patients' movement between APRI risk categories over time. Cut-offs used were <0.5 "low risk", 0.5-1.49 "indeterminate risk" and >1.5 "high risk". Very few patients moved from low to high risk over follow-up and 108 patients died.

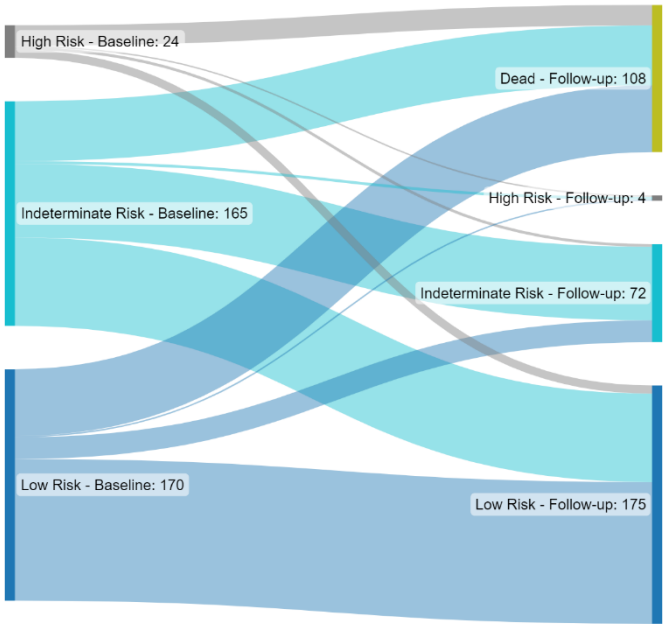


Figure 4.2. Sankey diagram demonstrating patients' movement between FIB-4 risk categories over time. Age corrected cut-offs were used, and these were <0.12 "low risk", 0.12-1.676 "indeterminate risk" and >1.676 "high risk". Very few patients moved from low to high risk over follow-up.

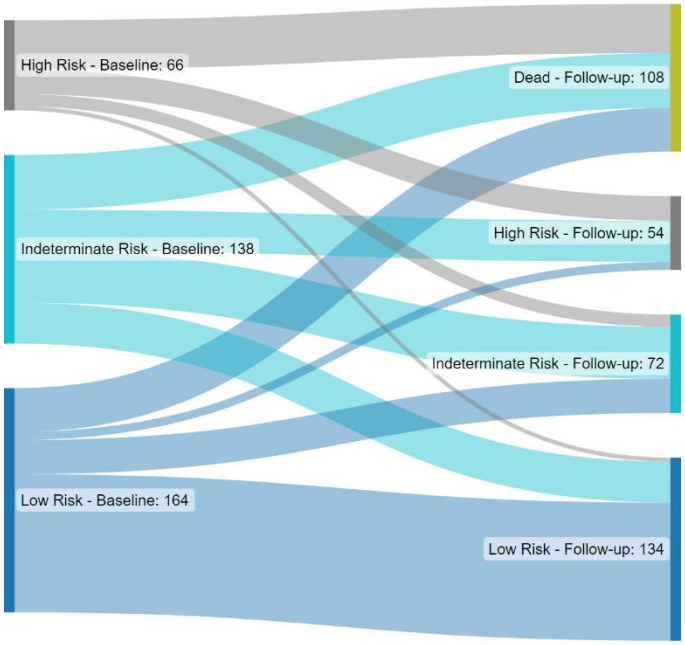
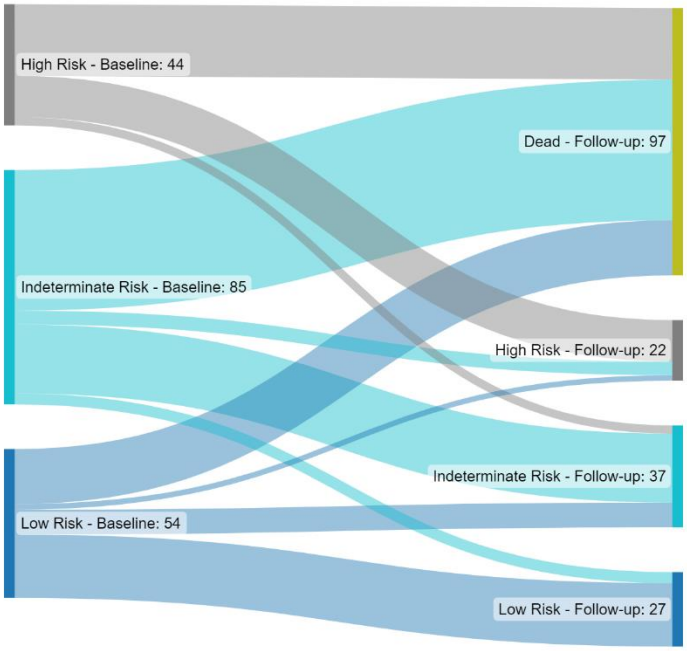


Figure 4.3. Sankey diagram demonstrating patients' movement between NFS risk categories over time. Age corrected cut-offs were used, and these were <2.0 "low risk", 2.0-2.67 "indeterminate risk" and >2.67 "high risk". Very few patients moved from low to high risk over follow-up.



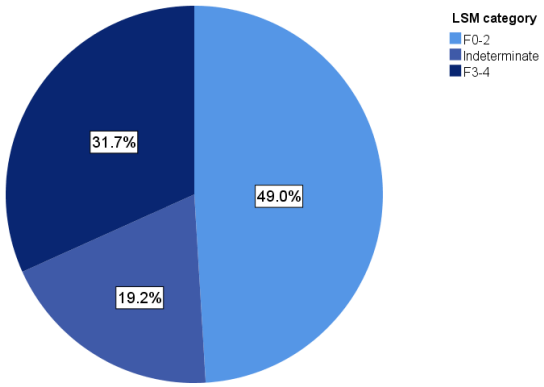
For those regularly attending clinical follow-up with the Hepatology Team, Fibroscan™ assessments are performed annually until a diagnosis of cirrhosis is made. One hundred and

four (17.2%) of the cohort had transient elastography at the final follow-up event and the median LSM result for the cohort was 8.3kPa, suggesting moderate fibrosis. The full results of the Fibroscan™ assessments are displayed in table 4.4 below. Figure 4.4 also shows the distribution of patients that fell into F0-2, indeterminate and F3-4 categories based upon the cut offs <8.0kPa, 8.0-11.99kPa and >12.0kPa as recommended by Cassinotto and colleagues in a NAFLD specific cohort.[192, 326]

Table 4.4. Follow-up Fibroscan™ results for Newcastle NAFLD cohort.

Fibroscan™	Result n=104
Median Liver Stiffness (kPa)	8.3 (2.6-75.0)
Inter-Quartile Range (kPa)	1.3 (0.1-13.9)
IQR/Med %	15.3 ± 6.7
CAP (dB/m)	317 (36-400)
Probe • M • XL	 50 (52.1%) 46 (47.9%)

Figure 4.4. Distribution of fibrosis stage based upon Fibroscan™ results at follow-up. Cut-offs used were <8.0kpa, 8.0-11.6kPa and >12.0kPa as recommended by Cassinotto et al in a NAFLD specific study.



It is important to note that once a patient has been diagnosed as having cirrhosis, and prior to the publication of the Baveno IV guidelines [338], there was no clinical indication to perform a Fibroscan™, the sole purpose of which is to clinically stage liver disease. At the time of final follow-up, a significant number of patients had been staged as cirrhotic and therefore a repeat Fibroscan™ would not have been performed.

4.3.3 Follow-up Histology

At the time of final follow-up only a very small group (22) of patients underwent repeat liver biopsies for further disease staging. Many patients had several clinical events in between the baseline and final, and a significant proportion of these interim events include repeat liver biopsies. As the aim of this chapter is to provide an overview of the outcomes of the cohort, the follow-up histology results will not be explored in detail.

4.4 Co-morbidities

As described in chapter three, the Newcastle cohort was shown to have many of the co-morbidities known to be associated with NAFLD. The incidence of these co-morbid conditions and clinical outcomes of interest at the time of the last clinical event are described below.

Patients who received a liver transplant during the follow-up period will remain classified as being diagnosed with cirrhosis for the following analysis.

4.4.1 Type 2 Diabetes Mellitus

By the time of final event follow-up, the number of patients with Type 2 Diabetes Mellitus had markedly increased to 71.8% (410) of the cohort, including 81 patients that had died. There were 123 new diagnoses of T2DM during the follow-up period.

Table 4.5 below shows a comparison of the characteristics of those with diabetes vs those without diabetes at the time of follow-up and explores any significant differences between the groups at the end of the study. Those with diabetes remained older (66 vs 60 years), with a higher BMI ($p=0.014$) and there continued to be a significantly higher proportion of diabetic patients with cirrhosis compared to non-diabetic patients ($p<0.001$). In contrast to baseline, there was no significant difference in albumin ($p=0.053$), platelets ($p=0.791$) or PT ($p=0.647$) between the groups but ALT and AST levels were significantly lower in those with diabetes ($p=0.006$, $p=0.020$ respectively).

Table 4.5. Comparison of demographic characteristics between diabetes groups at the last clinical event. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

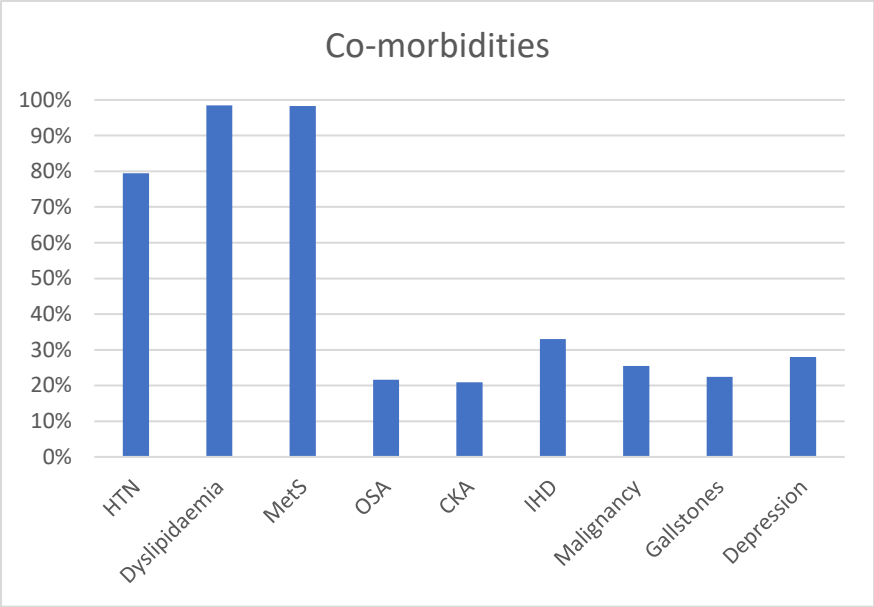
Variable	T2DM n=410	No T2DM n=161	p value
Age	66 (30-97)	60 ± 15	<0.001~
BMI (kg/m^2)	34.2 (23.5-52.5)	31.4 (23.5-45.2)	0.014~
HbA1c$^+$	58 (22-133)	39 (26-120)	<0.001~
Fasting glucose$^\#$	8.8 (4.5-41.6)	5.3 (4.0-11.0)	<0.001~
ALT (0 – 40) [U/l)	30 (7-232)	36 (10-314)	0.006~
AST (0 – 40) [U/l)	29 (12-156)	35 (14-680)	0.020~
GGT (0 – 70) [U/l)	61 (11-2002)	56 (10-1650)	0.729~
Albumin (35 – 50) [g/l]	45 (25-74)	46 (23-52)	0.053~
Platelets (150 – 450) [x10^9/l]	220 (24-648)	216 (81-452)	0.791~
PT (10 – 13) [seconds]	12 (9-49)	12 (10-41)	0.647~
IgA (0.8 – 3.0) [g/l]	2.68 (0.84-7.46)	2.37 (0.62-7.38)	0.478~
APRI	0.34 (0.06-2.08)	0.41 (0.14-4.99)	0.046~
FIB-4 Score	1.45 (0.34-8.37)	1.45 (0.39-8.30)	0.923~
NFS	0.11 ± 1.47	-1.39 ± 1.27	<0.001$^+$
Cirrhosis	137 (39.7%)	17 (11.6%)	<0.001$^\#$
~Mann Whitney U test, + Student T test $^\#$ Chi-squared $^+$HbA1c <42 mmol/mol = non-diabetes, >47mmol/mol diagnostic of diabetes. $^\#$ >7.0mmol/l = consistent with diabetes, 6.1-7.0mmo/l “impaired glucose handling”			

By the end of the study, there was no longer a significant difference in the mean results of the FIB-4 score between groups (p=0.923) but there was for APRI (p=0.046) and the NFS (p<0.001). Of note, the group of patients with diabetes had a lower APRI score than those without diabetes, which likely reflects the limitations of this non-invasive tool to risk assess advanced disease in an older population rather than this group having less advanced disease as this result might suggest.

On reviewing the proportion of patients in different risk categories using the non-invasive scores, and age-appropriate cut-offs, the NFS was the only tool with a significantly higher proportion of patients with diabetes in the high-risk category ($p<0.001$), which is unsurprising given the presence of diabetes is one of the components of the NFS score.

Patients with T2DM at the final clinical event were also noted to have a high prevalence of other co-morbidities, some of which are demonstrated in Figure 4.5 below. Almost all of those with T2DM also had Dyslipidaemia (98.5%, 270), 98.3% (294) met the criteria for the Metabolic Syndrome, as defined in chapter one[55], and 33.0% (106) had Ischaemic Heart Disease.

Figure 4.5. Presence of co-morbidities and clinical outcomes in those with T2DM at last follow-up.



HTN= hypertension, MetS= metabolic syndrome, CKD= chronic kidney disease, OSA= obstructive sleep apnoea.

4.4.2 Hypertension

The definition used for a diagnosis of HTN at follow-up remained the same as the baseline; a recorded systolic BP >140mmHg, diastolic BP >80mmHg, an existing clinical diagnosis and the use of anti-hypertensive medication.

The number of patients diagnosed with HTN markedly increased by the time of final follow-up event; 71.0% (401) of the cohort were hypertensive by this time, including 84 patients that had died. Those with hypertension remainder older (mean 68 vs 58 years), had higher mean BMI results ($p=0.006$) and there remained a significantly higher proportion of hypertensive patients with cirrhosis compared to the normotensive group ($p=0.030$). There was no longer any clinically significant difference in laboratory results between groups at this

time, and when exploring the differences in non-invasive staging scores there was a higher NFS score in those with HTN (p=0.007) but no difference in the proportion of patients across the risk categories for any of the scores (APRI p=0.78, FIB-4 p=0.518, NFS p=0.111).

4.4.3 Dyslipidaemia

The presence of dyslipidaemia was almost universal across the cohort by the time of the final clinical follow-up (96.2%) and therefore the value of analysis comparing those with dyslipidaemia to those without is limited due to significantly differing group sizes.

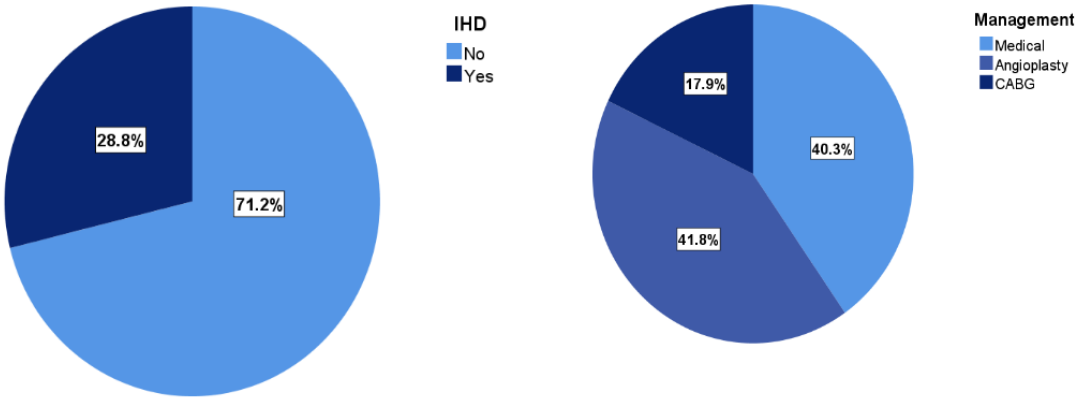
4.4.4 The Metabolic Syndrome

More than 90% of the cohort met the criteria for the diagnosis of the metabolic syndrome[55] by the end of the study (91.8%, 345). The mean BMI of this group remained higher than those who did not fulfil the diagnostic criteria (p=0.001), but there was no longer any significant difference in age (p=0.209) or proportion of patients with cirrhosis (p=0.278). However, given the number of patients that did not fulfil the criteria for MetS was so small by this stage it is hard to draw any meaningful conclusions from analysis of these data.

4.5 Clinical Outcomes

4.5.1. Ischaemic Heart Disease

Figure 4.6. Prevalence of IHD in the Newcastle NAFLD cohort at follow-up and management methods.



One hundred and thirty seven (28.8%) of the cohort had experienced a cardiovascular event by the time of the final follow-up event, including 55 patients who had died. Almost half, (65) of these were new diagnoses and the mean time to cardiac event was 109 ± 86 months (9.1 ± 7.2years).

Table 4.6 below demonstrates the differences between the patients who had a diagnosis of IHD at follow-up and those who did not. Those with IHD remained older (72 vs 63 years) and there was still no significant difference in mean BMI result (p=0.841) or sex (p=0.474) between the groups. There was no longer a significant difference in GGT (p=0.460) and platelet (p=0.222) results, but albumin levels remained lower in those with IHD (p=0.008).

Table 4.6. Comparison of demographic characteristics between IHD groups at the final event. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	IHD n=137	No IHD n=338	p
Age	72 (36-96)	63 (29-92)	<0.001^{+}
Sex (male)	84 (61.3%)	195 (57.5%)	0.474$^{\#}$
BMI (kg/m^2)	33.7 ± 4.6	32.8 (23.5-52.5)	0.841*
ALT (0 – 40) [U/l)	29 (7-197)	32 (8-314)	0.098*
AST (0 – 40) [U/l)	31 (12-94)	30 (14-680)	0.866*
GGT (0 – 70) [U/l)	82 (14-407)	58 (10-2002)	0.460*
Albumin (35 – 50) [g/l]	44 (25-53)	45 (23-74)	0.008*
Platelets (150 – 450) [x10^9/l]	211 (24-648)	219 (56-497)	0.222*
PT (10 – 13) [seconds]	14 ± 6	13 ± 4	0.091*
IgA (0.8 – 3.0) [g/l]	2.74 ± 0.89	2.53 (0.84-7.46)	0.964*
APRI	0.34 (0.09-1.25)	0.36 (0.10-4.99)	0.831*
FIB-4 Score	1.54 (0.46-5.23)	1.45 (0.34-8.37)	0.322*
NFS	0.02 (-3.32-2.73)	-0.34 ± 1.52	0.884*
Cirrhosis	53 (46.5%)	63 (18.8%)	<0.001$^{\#}$
*Mann Whitney U test, $^{\#}$ Chi-square, $^{+}$Student T test			

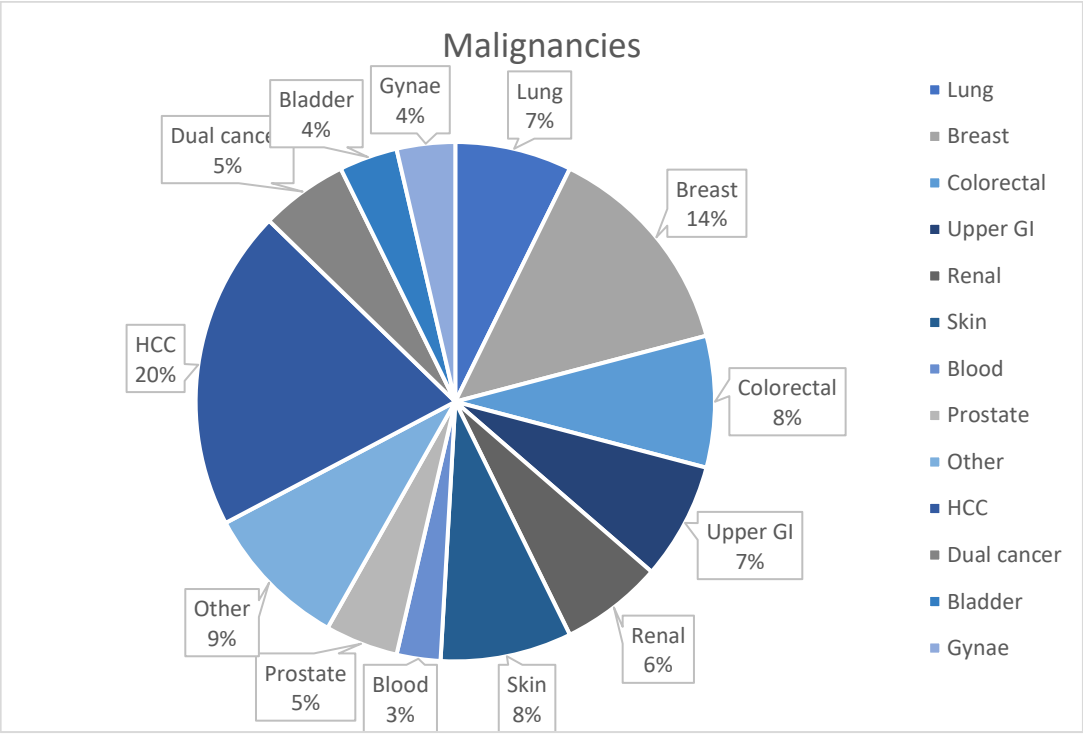
The incidence of co-morbidities in the group of those with IHD compared to those who did not have heart disease had also increased from the baseline event. Diabetes was present in 84% vs 64% (p<0.001), 84% vs 65% HTN (p<0.001), 99% vs 95% Dyslipidaemia (p=0.313) and 99% vs 88% met the criteria for MetS (p=0.005). The number of patients with IHD and cirrhosis had also increased to 47% of the group (53) by the end of follow-up, vs 19% in those with no heart disease (p<0.001).

When exploring the differences between the IHD groups at baseline in chapter 3 it was found that there was a significantly higher proportion of those who had a diagnosis of IHD that fell into the "high risk" category in the non-invasive scores. This was repeated using age adjusted repeat scores at the final clinical event and there was no longer a significant difference found in either mean score results or distribution across risk categories (APRI p=0.894, FIB-4 p=0.613, NFS p=0.893).

4.5.2. Malignancy

The incidence of malignancy had increased to 23.2% (111) of the cohort by the time of final follow-up event, including 56 patients that died over the course of the study. HCC was the most common malignancy by the final clinical event, accounting for 20% (22), and a small number of patients (5.5%, 6) had two separate occurrences of cancer. Figure 4.7 below displays the break-down of the different malignancies diagnosed. The "other" malignancy diagnoses included Ewing's Sarcoma, Parathyroid cancer, and Cholangiocarcinoma.

Figure 4.7. Detailed description of the categories of malignancy diagnosed in patients across the study.



The median time to cancer diagnosis from the baseline event was 8.3 years (1.0-35.4) and the median age of a new diagnosis of malignancy during follow-up was 66 years. Table 4.7 below details the different characteristics between the groups. Patients with a diagnosis of malignancy remained older (mean 73 vs 62 years) and as with baseline there was an increased association with co-morbidities such as diabetes (p=0.002) and HTN (p=0.004).

There was also a significantly higher proportion of cirrhosis in the group who had a cancer diagnosis; 53.3% (49) vs 20.8% (76).

Table 4.7. Comparison of demographic characteristics stratified by the presence of malignancy at the last follow-up. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

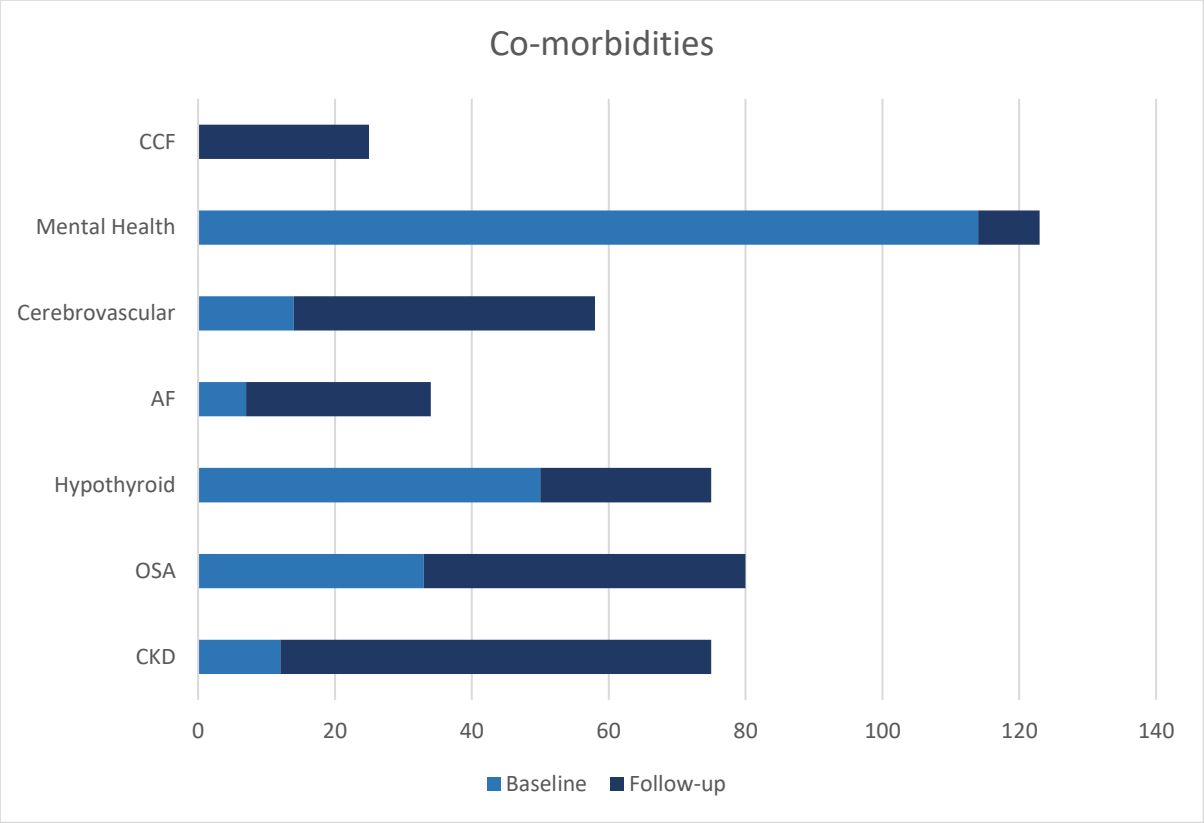
Variable	Malignancy n=111	No Malignancy n=367	p
Age	73 ± 9	62 ± 12	0.001*
BMI (kg/m^2)	34.1 ± 6.3	33.5 (23.5-52.5)	0.960~
ALT (0 – 40) [U/l)	28 (10-134)	32 (7-314)	0.056~
AST (0 – 40) [U/l)	32 (14-75)	30 (12-680)	0.657~
GGT (0 – 70) [U/l)	75 (19-751)	57 (10-2002)	0.334~
Albumin (35 – 50) [g/l]	43 (25-50)	45 (23-74)	<0.001~
Platelets (150 – 450) [x10^9/l]	161 (41-648)	237 ± 74	<0.001~
PT (10 – 13) [seconds]	13 (11-30)	12 (9-49)	<0.001~
IgA (0.8 – 3.0) [g/l]	2.53 (1.27-7.04)	2.55 (0.84-7.46)	0.474~
APRI	0.54 (0.16-1.25)	0.35 (0.09-4.99)	0.002~
FIB-4 Score	2.25 (0.67-53.0)	1.37 (0.34-8.37)	<0.001~
NFS	0.11 (-1.20-3.93)	-0.46 ± 1.55	0.009~
T2DM	83 (83.0%)	243 (66.6%)	0.002#
HTN	81 (82.7%)	244 (67.2%)	0.004#
IHD	40 (46.5%)	70 (19.6%)	<0.001#
Cirrhosis	49 (53.3%)	76 (20.8%)	<0.001#
*Student t-test, #Chi-square test, ~Mann Whitney U Test			

When comparing the proportion of patients in each risk category for the non-invasive tools there was a significantly higher proportion of patients with malignancy in the high-risk group for both the APRI (p=0.006) and FIB-4 scores (<0.001), however there was no difference in the NFS categories (p=0.357).

Of the 112 patients from the entire cohort that died during follow-up, 12.5% (14) died of HCC and 20.5% (23) died of another form of malignancy.

4.5.3. Other co-morbidities

Figure 4.8. Prevalence of "other" co-morbidities at follow-up and their increase in incidence over the duration of the study.



CCF= congestive cardiac failure, AF= atrial fibrillation, OSA= obstructive sleep apnoea, CKD= chronic kidney disease

Figure 4.8 above details the other co-morbidities present in the cohort at the time of the final clinical event.

A mental health diagnosis remained the most common "other" co-morbidity (29.2%), although only an additional 9 patients had been diagnosed with a new mental health condition since the baseline visit.

The incidence of CKD significantly increased over the follow-up period from 2.7% (12) to 17.6% (75) of the cohort. The relationship between CKD and T2DM was found to be significant (p=0.006) with 82.2% (60) of the patients diagnosed with CKD having a concurrent diagnosis of T2DM. In patients with CKD, 85.3% also had HTN (p=0.001) and 43.1% (28) had a history of ischaemic heart disease (p<0.001). Nineteen (28.8%) patients with CKD at follow-up had a diagnosis of cirrhosis at the final event, but this relationship was not found to be significant; p=0.263.

Atrial Fibrillation had increased from 1.5% (7) of the cohort at baseline to 8.3% (34) by the final clinical event and Congestive Cardiac Failure (CCF), which was not originally documented in the cohort, was present in 5.8% (25) by the end of the study. Of note 50% (11) of those diagnosed with CCF also had cirrhosis, which was found to be significant, p=0.005.

The diagnosis of OSA in the cohort increased from 6.6% (33) to 18.5% (80). A third, 33.9% (21), of those with OSA also had cirrhosis by the end of the study (p=0.032).

A cerebrovascular event had occurred in 13.0% (58) by the end of follow-up, the mean time to event was 136 ± 88 months (11.3 ± 7.3 years). As would be expected, given the underlying pathophysiology of CVA, there was a significant association with AF (p=0.016) and IHD (p=0.002). However, there was no significant relationship found between CVA and cirrhosis by the end of follow-up, p=0.171.

Many of these co-morbidities were associated with increasing age; CKD p=0.001, AF p=0.001, CCF p=0.001 and CVA p=0.001. However, it was surprising to observe there was no significant difference in mean BMI between groups with vs without each diagnosis; CKD p=0.462, AF p=0.870, CVA p=0.942, given it is widely accepted that each of these conditions are directly related to obesity. OSA however did not demonstrate a significant relationship with age, p=0.521, but did with BMI, p=0.021.

4.5.4 Bariatric Surgical Procedure

As was observed at the baseline visit, only a very small proportion of the cohort had undergone any bariatric procedure 14 (3.1%) by the final clinical event. Given these small numbers it is not possible to examine these data for meaningful associations with cirrhosis or other co-morbidities.

4.6 Liver Related Outcomes

4.6.1 Follow-up Cirrhosis

The proportion of patients with NASH cirrhosis by the time of the final clinical event had increased from 19.2% (116) at baseline to 32.9% (166) of the cohort due to 50 individuals progressing to cirrhosis during follow-up. This accounted for 10.2% of the original cohort, excluding those who were cirrhotic at baseline. The mean time to age of diagnosis of

cirrhosis was 120 ± 84 months (10 ± 7 years) from baseline biopsy and the mean age was 64 ± 11 years.

The vast majority of these were diagnosed radiologically with imaging (46%, 23) or elastography (12%, 6), whilst 30% had repeat histology confirming cirrhosis and 12% (6) had clinically decompensated leading to diagnosis. The features of those with cirrhosis by the end of the study will be further discussed in chapter 6.

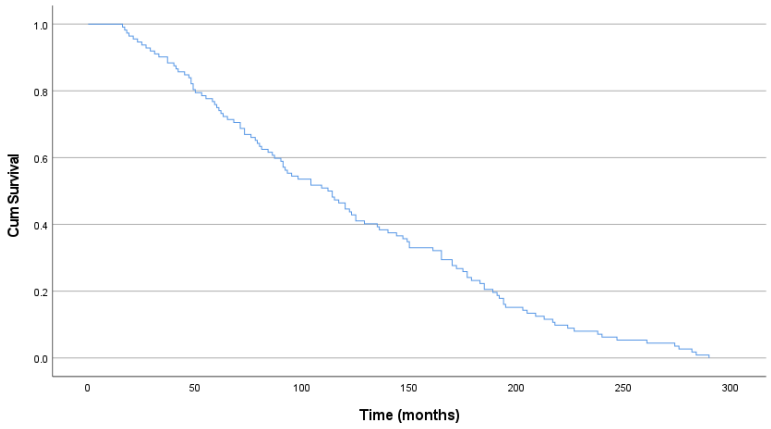
4.6.2 Liver Transplant

Only 1.7% (10) of the cohort had undergone liver transplantation by the time of the final clinical event, which accounted for 8.6% of those diagnosed with cirrhosis at baseline. Four (44.4%) of these developed an HCC which was the indication for transplant and 5 (55.6%) were listed for decompensation and deranged synthetic function. The mean time to transplant was 44 ± 42 months (3.7 ± 3.5 years). The group of patients that underwent a liver transplant during the study will be further explored in chapter 6.

4.7 Mortality

During the follow-up period of the study 18.5% (112) of the cohort died. The mean age at time of death was 74 ± 10 years and the mean time to death was 9.4 years (1.3-24.2). Of those who died 49.1% (55) were men and, in keeping with the majority ethnicity of the cohort, 98.1% were Caucasian. Figure 4.9 below displays time to death in a Kaplan Meier Survival curve for all-cause mortality.

Figure 4.9. Kaplan Meier survival curve demonstrating time to death for all-cause mortality of the Newcastle NAFLD cohort, in months. The median time to death was 9.4 years.



4.*7.1. Cause of death; all-cause mortality.*

The cause of death as documented on death certification was collected and the results are shown in table 4.8 below. The cause of death was categorised below using the diagnosis from section Ia, b or c, for example a death documented as “Decompensated NASH Cirrhosis” in section I of the certificate would be classified as a Cirrhosis cause of death and “Myocardial Infarction” would be Cardiovascular.

Table 4.8 Causes of death in the Newcastle NAFLD cohort as documented in section Ia, b or C of death certificate.

Cause of Death	Number of Patients (n=112)
Liver related • Cirrhosis • HCC	32 (28.6%) • 18 (16.1%) • 14 (12.5%)
Cardiovascular	23 (20.5%)
Extrahepatic Malignancy	23 (20.5%)
Other (1)	33 (29.5%)
Missing	1 (0.9%)

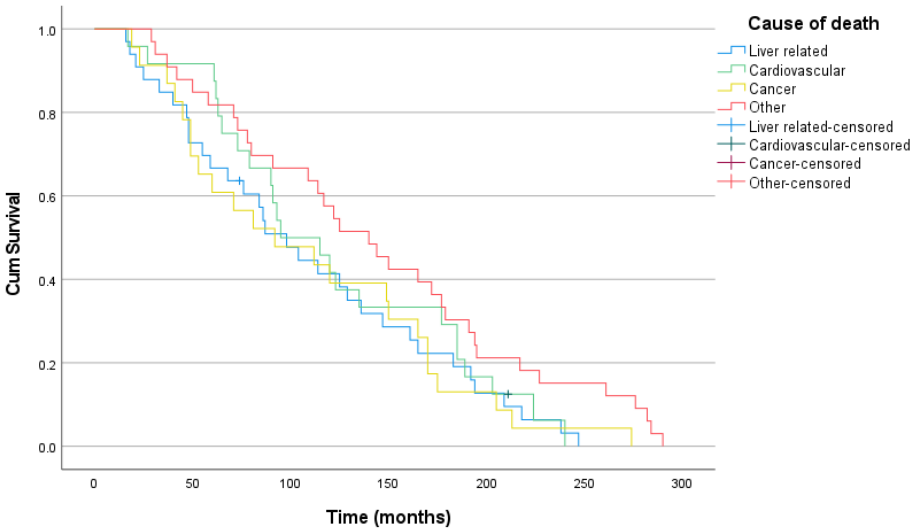
(1) For example, Respiratory disease, Infection, Frailty and Renal failure.

Of those who died of Malignancy, the most common was lung cancer (30.4%) followed by oesophageal (17.4%), gastric (8.7%) and breast cancer (8.7%).

The most common "other" cause of death was infection (33.3%) and 72.7% of these infections were pneumonia. Frailty of old age and dementia diagnoses were the second most common "other" cause of death accounting for 21.2%.

Figure 4.10 below shows time to death in a Kaplan Meier Survival curve broken down by cause of death as detailed above.

Figure 4.10. Kaplan Meier survival curve demonstrating time to death stratified by causes of death.



In order to gain a better understanding of any factors which may be associated with or predict mortality in the Newcastle NAFLD cohort, further examination of features present in this group at the time of the baseline visit was undertaken.

4.7.2. Baseline characteristics of patients who died compared to those alive.

The baseline characteristics of the group that died of any cause during the study were further explored and compared to those who survived by univariate analysis to explore any factors associated with an increased risk of mortality. As stated above, 49.1% (55) of those who died were men and the mean age at baseline was 60 ± 9 years old, which was significantly older than those who survived (52 years, $p<0.001$). Whilst ~50% of those that died were male there was a trend towards a difference in the sex of those who died ($p=0.072$) as proportionally there were more males in the entire study cohort (57% vs 43%) indicting a higher representation of female deaths by the end of the study.

Table 4.9 below displays the baseline demographic, liver related laboratory results and co-morbidity characteristics of the group who had died compared to those who survived.

The group of patients who died during the follow-up period had significantly lower mean ALT ($p=0.001$), Albumin ($p<0.001$) and Platelet levels ($p<0.001$) at baseline when compared to the group still alive. Of interest this group also had a significantly higher GGT at baseline ($p=0.012$). As may be expected, there was a significant increase in the proportion of the deceased patients who had associated co-morbidities including T2DM (61.3% vs 44.3%, $p=0.002$) and HTN (72.1% vs 50.1%, $p=0.001$), and clinical outcomes of interest such as IHD (37.5% vs 12.2%, $p<0.001$), malignancy (15.7% vs 5.1%, $p=0.003$) and cirrhosis (43.8% vs 13.6% $p<0.001$) at the time of the baseline visit.

Table 4.9. Comparison of baseline demographic characteristics between those who were alive at the end of the study vs those who had died. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Alive n=493	Dead n=112	p value
Duration of follow-up (months)	152 (12-425)	113 (16-290)	0.016^{+}
Age (years)	52 (18-82)	60 ± 9	<0.001^{+}
Sex (male)	290 (58.8%)	55 (49.1%)	0.072*
BMI (kg/m^2)	34.0 (22.7-59.1)	34.7 ± 5.1	0.658^{+}
ALT (0 – 40) [U/l)	62 (10-355)	50 (13-278)	<0.001^{+}
AST (0 – 40) [U/l)	43 (13-251)	41 (16-225)	0.712^{+}
GGT (0 – 70) [U/l)	79 (14-2062)	101 (20-691)	0.012^{+}
Albumin (35 – 50) [g/l]	45 +/- 3	43 (27-52)	<0.001^{+}
Platelets (150 – 450) [x10^9/l]	239 (71-498)	203 ± 78	<0.001^{+}
PT (10 – 13) [seconds]	12 (9-19)	12 (10-18)	<0.001^{+}
IgA (0.8 – 3.0) [g/l]	2.61 (0.42-20.90)	3.89 (0.1-12.90)	<0.001^{+}
T2DM	218 (44.3%)	68 (61.3%)	0.002*
HTN	227 (50.1%)	49 (72.1%)	0.001*
MetS	273 (73.4%)	53 (77.9%)	0.457*
IHD	48 (12.2%)	24 (37.5%)	<0.001*
Malignancy	23 (5.1%)	11 (15.7%)	0.003$^{\#}$
Cirrhosis	67 (13.6%)	49 (43.8%)	<0.001*
$^{+}$Mann Whitney U, *Chi square test, # Fisher's Exact			

Of the 49 patients who died that were diagnosed with cirrhosis at the time of baseline, the majority were diagnosed by liver biopsy (65.3%, 32). Of the remaining cases, 26.5% (13) were clinically diagnosed and 8.1% (4) by imaging modalities, including CT, or elastography.

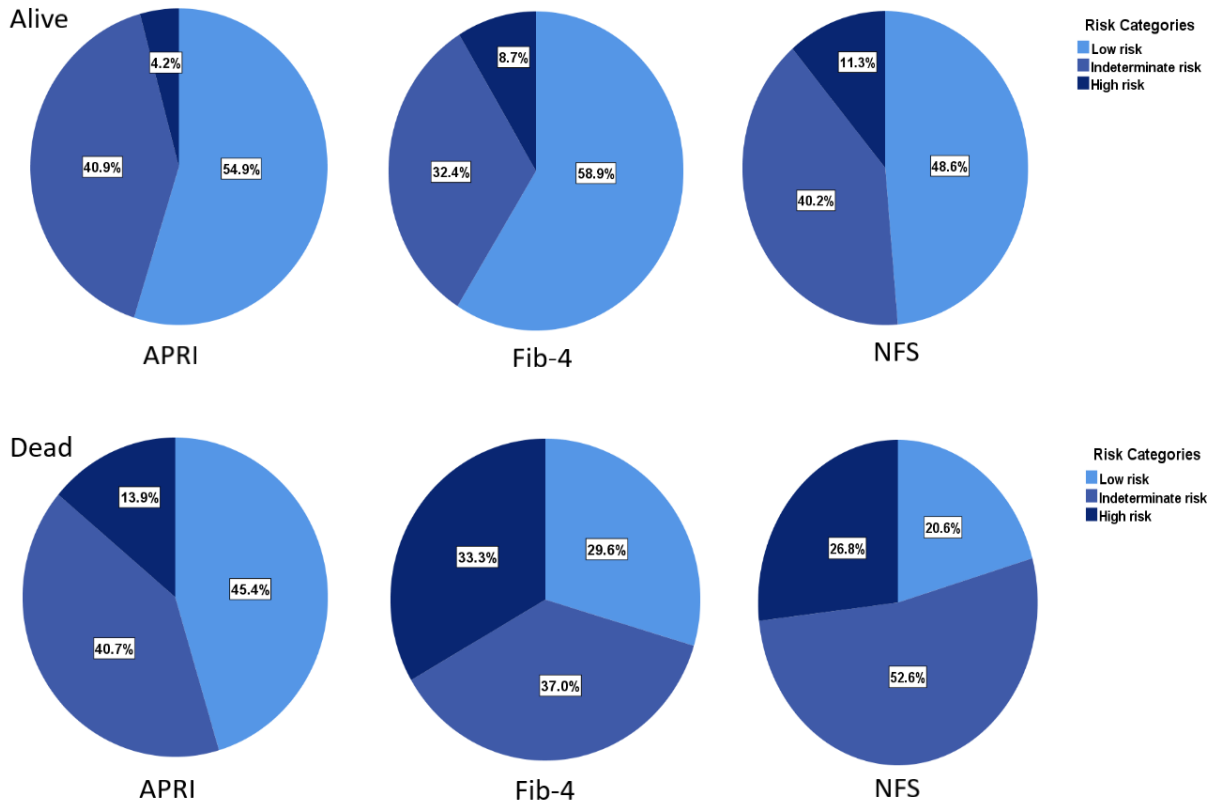
Whilst the non-invasive disease staging tools were developed to predict advanced liver disease and not mortality, it has been shown that advanced fibrosis is a predictor of mortality outcomes and therefore a comparison of the results of the APRI, FIB-4 and NFS between the mortality groups was explored. The median result of each score at baseline was

significantly higher in the group who died (APRI p=0.006, FIB-4 p<0.001 and NFS p<0.001) as can be seen from table 4.10 below, and there were significantly more patients in the indeterminate and high-risk categories when compared to those still alive. Figure 4.11 also demonstrates the distribution of risk between the groups.

Table 4.10. Comparison of baseline non-invasive staging tool results between those alive at the end of follow-up vs those who had died. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Non-invasive Tool	Alive n=493	Dead n=112	p value
AST/ALT Ratio	0.71 (0.22-2.91)	0.97 (0.40-2.54)	<0.001[+]
APRI	0.47 (0.08-3.18)	0.64 (0.11-3.97)	0.006[+]
FIB-4 Score	1.16 (0.15-7.34)	1.92 (0.53-12.75)	<0.001[+]
NFS	-1.39 +/- 1.73	-0.06 ± 1.68	<0.001[+]
Child's Pugh Score	5 +/- 1	6 ± 1	0.008[+]
[+]Mann Whitney U			

Figure 4.11. Proportion of patients in each risk category by non-invasive tool at baseline stratified by death by the end of study. Cut offs used were APRI 0.5 and 1.5, FIB-4 1.30 and 2.67, and NFS -1.455 and 0.676.



Ninety five (84.8%) of those who died by follow-up had a liver biopsy performed at the time of inclusion to the study. The results of the baseline biopsies for this group are detailed in table 4.11 below and compared to those who survived. Almost half (48.4%, 46) of those who died had advanced fibrosis (F3 or 4), 58.9% (56) had NASH and 46.3% (44) met the criteria for fibrosing steatohepatitis on histology. In keeping with what has thus far been described in the literature, the only significant difference in baseline histology between the groups was the presence of more advance fibrosis in those that died.

Table 4.11. Comparison of baseline histology between survival groups as defined by the NASH CRN criteria. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	Alive n=481	Dead n=112	p value
Steatosis • 0 • 1 • 2 • 3	 1 (0.2%) 144 (29.9%) 216 (44.9%) 120 (24.9%)	 3 (3.2%) 30 (31.6%) 43 (45.3%) 19 (20.0%)	0.198*
Ballooning • 0 • 1 • 2	 166 (34.5%) 214 (44.5%) 101(21.0%)	 32 (33.7%) 39 (41.1%) 24 (25.3%)	0.568*
Inflammation • 0 • 1 • 2 • 3	 144 (23.7%) 218 (45.3%) 138 (28.7%) 11 (2.3%)	 25 (26.3%) 46 (48.4%) 24 (25.3%) 0	0.264*
Fibrosis • 0 • 1 • 2 • 3 • 4	 133 (27.7%) 96 (20.0%) 107 (22.2%) 101 (21.0%) 44 (9.1%)	 17 (17.9%) 15 (15.8%) 17 (17.9%) 17 (17.9%) 29 (30.5%)	<0.001*
Fibrosis category • No significant • Clinically significant • Advanced	 229 (47.9%) 252 (52.4%) 145 (30.1%)	 32 (33.7%) 63 (66.3%) 46 (48.4%)	 0.013# 0.001#
NASH	62.2% (301)	58.9% (56)	0.563#
NAS Score Category • 1-2 • 3-4 • ≥5	 122 (25.4%) 168 (34.9%) 191 (39.7%)	 25 (26.3%) 36 (37.9%) 34 (35.8%)	0.565*
SAF Score	2 (0-4)	2 (0-4)	0.864*
Fibrosing steatohepatitis	45.3% (218)	46.3% (44)	0.910#
*Kruskal Wallis, #Chi square			

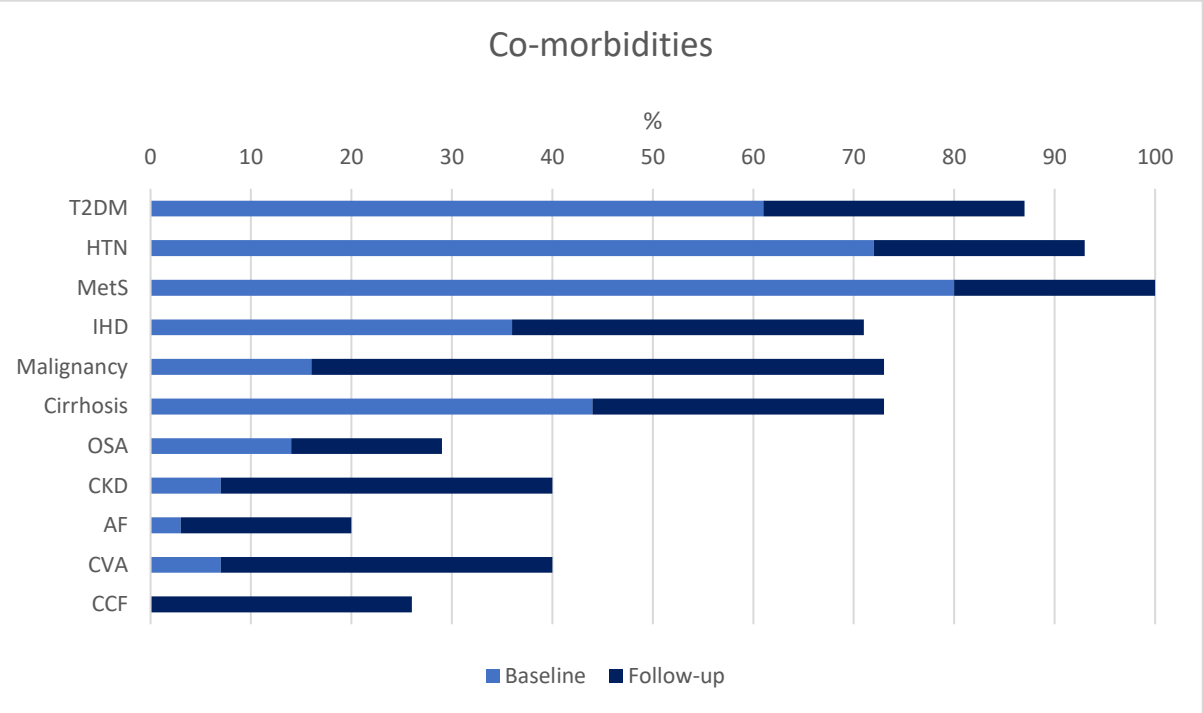
4.7.3. End of study characteristics of those that died.

One of the limitations of this study was the ability to access up-to-date medical records of the patients that died during the follow-up period. There were a number of those who died

that were no longer under the care of the Newcastle Hospitals and therefore it was not possible to obtain detailed information regarding the diagnosis of new co-morbidities and clinical outcomes of interest in the time between their initial clinical visit and death.

From the data that was available, in keeping with the overall cohort, there had been an increase in the prevalence of many of co-morbid conditions of interest such as T2DM, IHD, CKD and Stroke. Figure 4.12 below demonstrates the change in proportion of the co-morbidities of interest with time from the available data.

Figure 4.12. Proportion of co-morbidities in all-cause mortality group at the time of death and the increase in incidence over the follow-up period.



There is limited value in extensive analysis of the end of study characteristics of this group compared to those who were alive at the end of the study, however, as may well be expected there were clear trends in increased proportions of co-morbidities in those who had died. For example, T2DM was present in 87.1% vs 68.8% which was found to be statistically significant [p<0.001 OR 3.06 (95% CI 1.61 - 5.78)]. Similarly, there were significant differences when comparing each of the above diseases: HTN p<0.001, IHD p<0.001, CKD p<0.001 and CVA p<0.001 to demonstrate a few.

These results are in keeping with known risk factors for death in any patient population, including the NAFLD population, so whilst increased prevalence of co-morbid conditions and

clinical outcomes is expected with time they should be taken into consideration when managing these patients as higher risk for death in a clinical context.

4.8 Predictors of all-cause mortality

Multivariate analysis of the significant variables from baseline univariate tests was then performed to further explore any factors at baseline which may predict all-cause mortality. Any variable which met the univariate significance of $p \leq 0.100$ was considered for analysis at this stage, but only results with $p \leq 0.05$ were considered significant in predicting mortality.

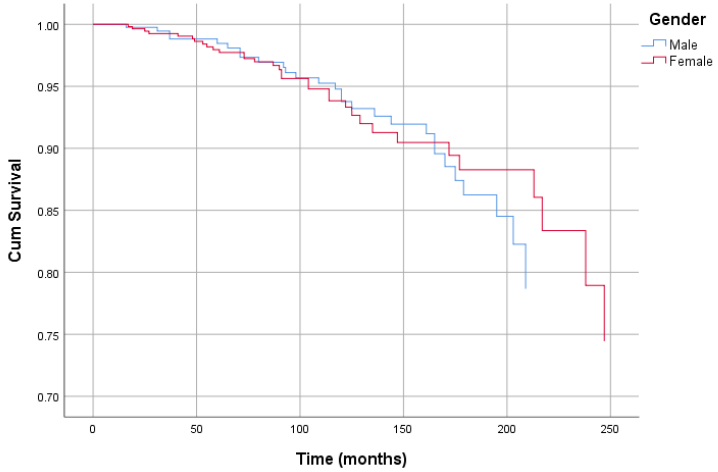
The analysis was divided into different categories of variables which would have the most relevance in a clinical setting: blood results, non-invasive scoring tools, clinical outcomes, and histology. This also enabled collinearity of results to be considered for example ALT, Age and Platelets all contribute to FIB-4 score and therefore these cannot be compared in the same model.

4.8.1 Anthropometric and Laboratory predictors

Multivariate Cox Regression modelling was used to review the routine liver blood tests available in a clinic setting which had been found to be significant by univariate analysis. A backwards stepwise Cox regression model was performed to examine the following variables: Age, sex, ALT, GGT, Albumin, Plt, PT and IgA.

When testing proportionality assumptions of the variable “sex” prior to its addition to the model, it was found to be non-proportional, which would mean the possible over estimation of risk with time. Sex was therefore not included as a covariate in the model, and instead was added as a stratum to explore its impact on the other covariates. As can be seen from figure 4.13 below, there was no significant difference between the sex groups, and it was therefore removed from the model altogether.

Figure 4.13. Sex as a stratum of laboratory and anthropometric predictors of mortality in Cox Regression. The graph below represents the effect of sex added to Cox regression models exploring blood results as predictors of mortality, demonstrating no significant difference between the different sexes.



At this stage, several variables were no longer significant in contributing as a control and they were therefore removed from the model. The variables removed were PT (p=0.627), ALT (p=0.623) and finally GGT (p=0.495). The model was then repeated, and IgA was found to no longer being significant at this stage (p=0.438). Table 4.12 below shows the results of the remaining variables that were shown to be independent predictors for mortality.

Table 4.12. Baseline blood tests which predict mortality by multivariate Cox proportionate hazard modelling. Models were created using results found to be significant at univariate analysis (age, sex, ALT, GGT, albumin, platelets, PT and IgA). These were added to a Cox regression model using backward logistic regression and age, albumin and platelets were found to be significant independent predictors of outcomes in the Newcastle NAFLD cohort.

Variable	aHR (95% CI)	p
Age	1.08 (1.06, 1.10)	<0.001*
Albumin	0.88 (0.84, 0.92)	<0.001*
Plt	0.995 (0.992, 0.998)	0.003*
*Multivariate Cox Regression		

Of note both Albumin and Platelet count appear to have a “protective” hazard ratio (<1.00). This is likely due to the known association of falling Albumin and Platelet levels being an indicator of advanced liver disease, and therefore higher baseline blood results of these tests could be interpreted as a protective factor against mortality.

The use of the AST/ALT ratio result was also trialled in the model in place of ALT, but this was not found to be significant.

4.8.2 Non-invasive tools as predictors of all-cause mortality

The non-invasive tools were also further examined to explore their ability to predict all-cause mortality. These tools have multiple shared variables contributing to their overall calculation (ALT, AST, Age, Platelets) and therefore they could not be forced into a regression model together due to collinearity.

Kaplan Meier survival curves were created for each of the non-invasive tools comparing each tool by risk categories using the log-rank test. There was a significantly higher mortality in the high-risk groups for each score (p<0.001 for each tool). Figures 4.14 – 4.16 below show the differences in survival for each category across each non-invasive tool.

Figure 4.14. Kaplan Meier survival curves of all-cause mortality stratified by APRI risk categories at baseline. Cut-offs used were <0.5 "low risk", 0.5-1.49 "indeterminate risk" and >1.5 "high risk". From the graph it can be seen that those in the "high-risk" group had an increased risk of mortality and shorter survival time than those in the "low-risk" group.

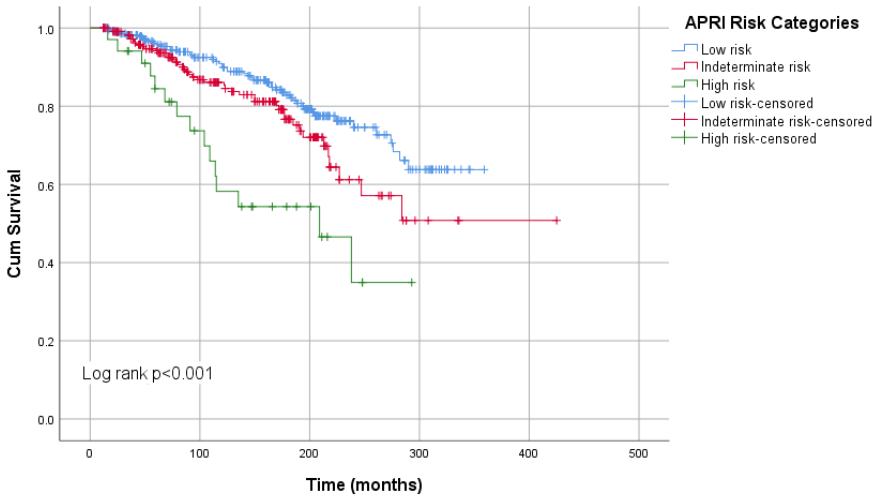


Figure 4.15. Kaplan Meier survival curves of all-cause mortality stratified by FIB-4 risk categories at baseline. Cut-offs used were <1.30 "low risk", 1.30-2.67 "indeterminate risk" and >2.67 "high risk". From the graph it can be seen that those in the "high-risk" group had a significantly increased risk of mortality (p<0.001) and shorter survival time than those in the "low-risk" group.

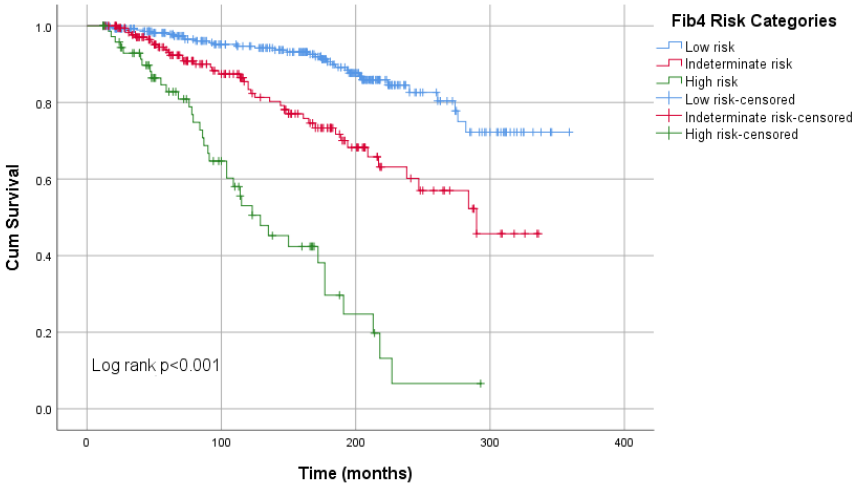
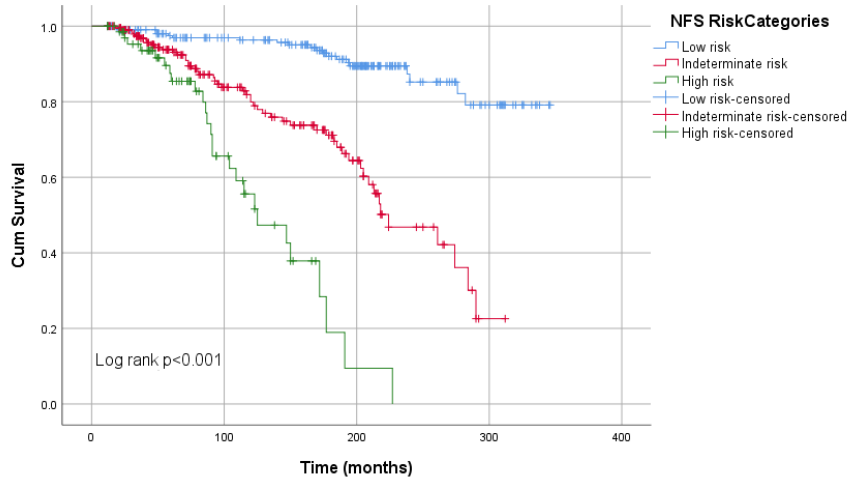


Figure 4.16. Kaplan Meier survival curves of all-cause mortality stratified by NFS risk categories at baseline. Cut-offs used were <-1.455 "low risk", -1.455-0.676 "indeterminate risk" and >0.676 "high risk". From the graph it can be seen that those in the "high-risk" group had a significantly increased risk of mortality (p<0.001) and shorter survival time than those in the "low-risk" group.



Hazard ratios for each tool were generated by univariate Cox Regression modelling for both mean scores but also results grouped by the risk categories and the results for this are shown in table 4.13 below. The results for the risk categories refer to comparison with "low risk" as the reference point. It is important to note that the risk category variables for each score, as with sex above, were found to be "non-proportional". There is therefore potential overestimation of the relative risk of HRs over time.

Table 4.13. Cox Regression hazard ratios of baseline non-invasive tools predicting all-cause mortality. Using "low-risk" as the comparator reference for each score, and the cut-offs as described throughout (APRI <0.5, 0.5-1.5, >1.5. FIB-4 <1.30, 1.30-2.67, >2.67. NFS <-1.455, -1.455-0.676, >0.676.), the table below again shows patients with "high-risk" scores at baseline have an increased risk of all-cause mortality and the hazard ratios associated.

Variable	HR (95% CI) -score means	p	HR (95% CI) -categories	p
AST/ALT Ratio	3.77 (2.57, 5.52)	<0.001	-	-
APRI • Low (ref) • Indeterminate • High	2.01 (1.52, 2.67)	<0.001	 1.49 (0.99, 2.25) 3.43 (1.92, 6.13)	 0.056 <0.001
FIB-4 Score • Low (ref) • Indeterminate • High	1.60 (1.48, 1.74)	<0.001	 2.78 (1.74, 4.43) 10.02 (6.14, 16.35)	 <0.001 <0.001
NFS • Low (ref) • Indeterminate • High	1.71 (1.55, 1.89)	<0.001	 5.35 (3.17, 9.06) 16.07 (8.57, 30.12)	 <0.001 <0.001
CP Score	1.35 (1.06, 1.72)	0.014	-	-

The "high-risk" NFS category group were shown to have the highest risk of mortality with a HR 16.07 (95% CI 8.57 – 30.12), but across each of the tools the "high-risk" category was associated with the highest risk of mortality.

The non-invasive tools were designed to identify patients with advanced fibrosis, and it is therefore logical that there is a significant association with the "high risk" category patients and mortality, as seen in the Cox regression, as advanced fibrosis has long been established as the biggest predictor of mortality in NAFLD.

4.8.3 Co-morbidities of interest as predictors

Baseline presence of co-morbidities which were significant by univariate analysis: T2DM and HTN, were also further explored to assess their ability to predict all-cause mortality. Kaplan Meier survival curves with log-rank test were performed before multivariate Cox Regression modelling to control for the influence of other variables associated with these conditions.

Figures 4.17 & 4.18 below demonstrate the Kaplan Meier survival curves of each of the co-morbid factors. Those with T2DM and HTN can clearly be seen to have an increased mortality, p<0.001 for both.

Figure 4.17. Effect of T2DM on Kaplan Meier survival curves in all-cause mortality. The curves demonstrate that patients with a diagnosis of T2DM at baseline are associated with an increased mortality and shortened survival time than those without diabetes (p<0.001).

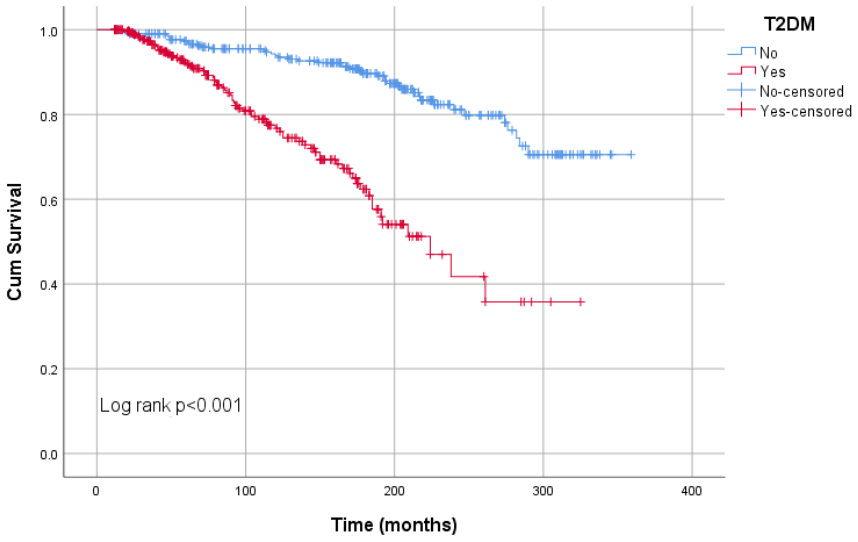
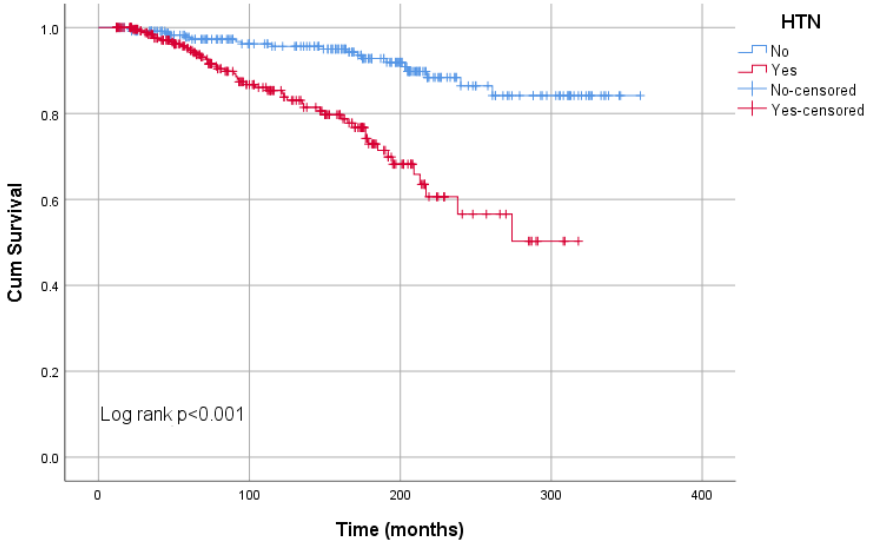


Figure 4.18. Effect of baseline HTN on Kaplan Meier survival curves in all-cause mortality. The curves demonstrate that patients with a diagnosis of HTN at baseline are associated with an increased mortality and shortened survival time than those without (p<0.001).



Proportionality of these categorical variables was assessed and again showed that both T2DM and HTN failed the assumptions of proportionality. In order to calculate hazard ratios for these variables they were used as covariates in Cox regression modelling, and it is therefore important to note that these HRs may be over-estimated with time.

When generating Cox regression models age and BMI were included as a covariate control as they had both been found to have a significant relationship with the co-morbidities and the effect of sex was also explored as a stratum but not found to be significant in controlling for any of these factors as can be seen from figure 4.19 below.

Figure 4.19. Sex as a stratum in Cox Regression models for T2DM and HTN. The graphs below show that the addition of gender to cox regression models exploring T2DM and HTN as predictors of mortality had no significant impact and therefore this was not used as a control in the models.

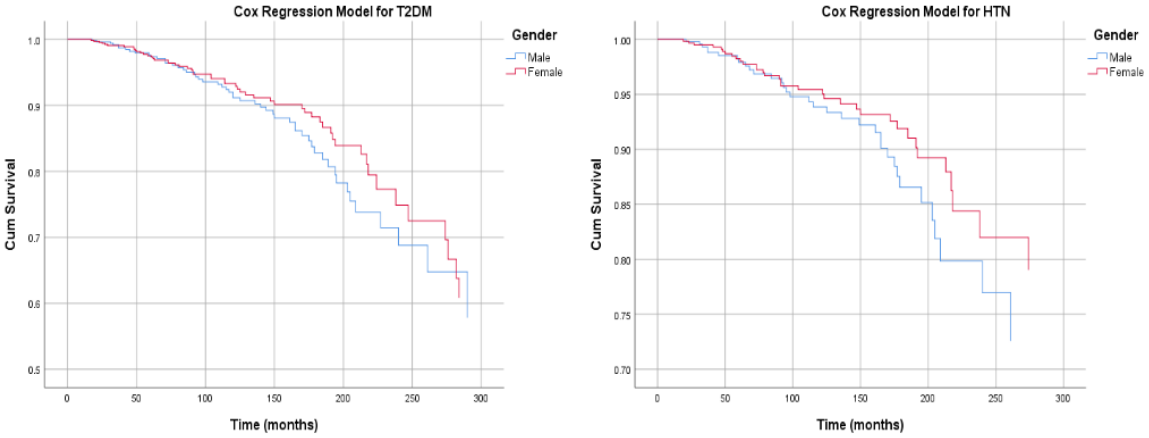


Table 4.14 below displays the results of the multivariate cox regression analysis. After controlling for age and BMI, T2DM was found to be the most significantly associated with mortality of the co-morbidities: aHR 2.48, p<0.001. HTN also was shown to be an independent predictor (p=0.012).

Table 4.14. Baseline co-morbidities which predict mortality by Cox Regression. Models were created exploring T2DM and HTN as predictors of mortality after these were found to be significant in univariate analysis. These factors were tested individually and then controlled for by age and BMI, and even after controls both T2DM and HTN were significantly associated with an increased risk of mortality in the Newcastle NAFLD cohort, p<0.001 and p=0.012 respectively. The table below demonstrates the hazard ratios generated from these models.

Variable	KM log rank p	HR (95%CI)	aHR (95%CI)	p
T2DM	<0.001	3.97 (2.66 - 5.92)	2.33 (1.51 - 3.60)*	<0.001
HTN	<0.001	3.90 (2.28 - 6.69)	2.03 (1.17 - 3.52)#	0.012
*Adjusted for age and BMI, #Adjusted for age only (BMI not significant)				

Fibrosis stage was found to be non-proportional on assessment and therefore could not be added into the model to control for any affect fibrosis may have had on the above co-morbidities. However, the variable advanced fibrosis (F3-4) was proportional and could therefore be added to control for the presence of advanced fibrosis and any affect this may

have. When added to the model HTN was no longer a significant independent predictor of all-cause mortality (p=0.277), however T2DM remained an independent predictor with aHR 1.98 (95% CI 1.25 – 3.14, p=0.004).

4.8.4 Clinical outcomes of interest to predict all-cause mortality.

The clinical outcomes of interest: IHD and malignancy were similarly explored using Kaplan Meier log rank tests then Cox regression multivariate analysis to assess their hazard ratios for all-cause mortality. Figures 4.20 & 4.21 demonstrate the Kaplan Meier survival curves for each of the clinical outcomes. As may be expected there is a clear increase in mortality in those patients with IHD and malignancy at baseline compared to those without (p<0.001 for both outcomes).

Figure 4.20. Effect of IHD at baseline on Kaplan Meier survival curves in all-cause mortality. The curves demonstrate that patients with a diagnosis of IHD at baseline are associated with an increased mortality and shortened survival time than those without (p<0.001).

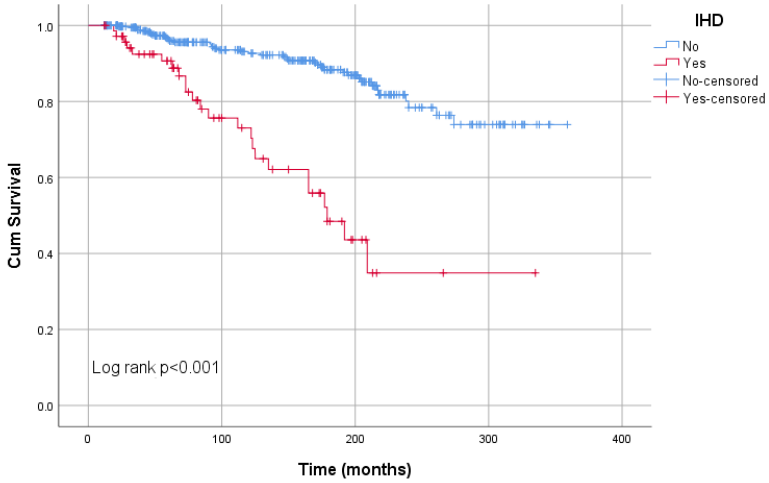
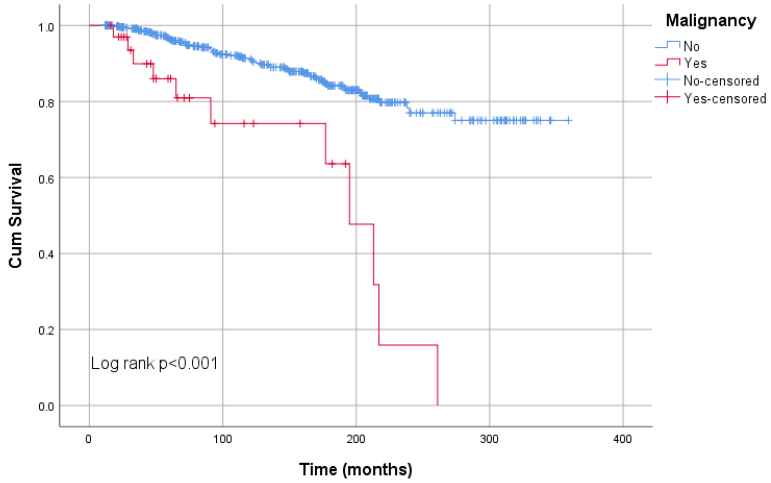
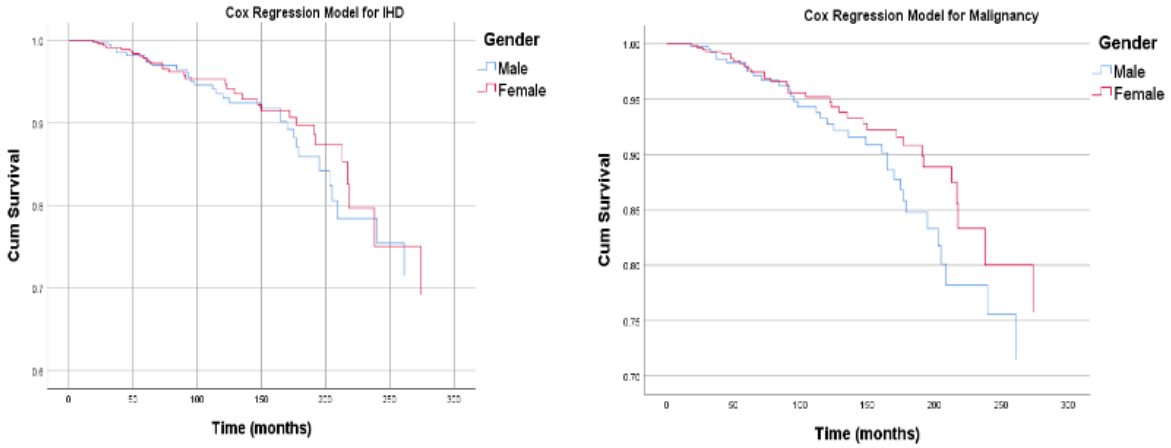


Figure 4.21. Effect of a diagnosis of malignancy at baseline on Kaplan Meier survival curves in all-cause mortality. The curves demonstrate that patients with a diagnosis of cancer at baseline are associated with an increased mortality and shortened survival time than those without (p<0.001).



These clinical outcomes were again explored for proportionality and were both found to be proportional. They were therefore able to be included in Cox proportional hazard models without risk of overestimation of their effect with time. As with the co-morbidities above age was added into the model as a control. Once again sex was added as a stratum and not found to be a significant influence on the outcomes as can be seen from figure 4.22 below.

Figure 4.22. Sex as a stratum in Cox Regression models for IHD and malignancy. The graphs below show that the addition of gender to cox regression models exploring IHD and cancer as predictors of mortality had no significant impact and therefore this was not used as a control in the models.



The presence of IHD at baseline was shown to be a significant independent predictor of mortality (aHR 2.44, 95% CI 1.41 – 4.21, p=0.001) but of interest malignancy at baseline was no longer significant after adjusted for Age and BMI controls (p=0.055). Table 4.15 below shows the hazard and adjusted hazard ratios for the clinical outcomes of interest.

Table 4.15. Baseline co-morbidities which predict mortality by Cox Regression. Models were created exploring IHD and malignancy as predictors of mortality after these were found to be significant in univariate analysis. These factors were tested individually and then controlled for by age and BMI, and even after controls IHD was significantly associated with an increased risk of mortality in the Newcastle NAFLD cohort, p=0.001, whereas malignancy was no longer significant. The table below demonstrates the hazard ratios generated from these models.

Variable	KM log rank p	HR (95%CI)	aHR (95%CI)[#]	p
IHD	<0.001	4.97 (2.96 - 8.32)	2.44 (1.41 – 4.21)	0.001
Malignancy	<0.001	4.71 (2.46 - 9.00)	1.93 (0.99 - 3.79)	0.055
*Adjusted for age only. #Adjusted for age and BMI.				

Once again, the presence of advanced fibrosis was controlled for in a repeat model and IHD remained an independent predictor of all-cause mortality aHR 2.31 (95% CI 1.27 – 4.20, p=0.006).

4.8.5 Histology as a predictor of all-cause mortality

Finally, the baseline histology markers were further examined to explore their association with all-cause mortality in the cohort. Fibrosis stage and fibrosis categories were the only significant variables found by univariate analysis, and therefore these were the only histological features explored. Kaplan Meier survival curves and log rank tests were performed for both variables and as above the log rank results for fibrosis stage are displayed as overall comparisons (i.e., F0 vs F4). From the figures below it can clearly be seen that the presence of F4 at baseline was associated with an increased in overall mortality.

Figure 4.23. Effect of baseline Fibrosis stage on Kaplan Meier survival curves in all-cause mortality. From these curves it can clearly be seen that those with F4 at baseline have a much shorter survival time and increased risk of mortality in comparison to F0 at baseline, p<0.001.

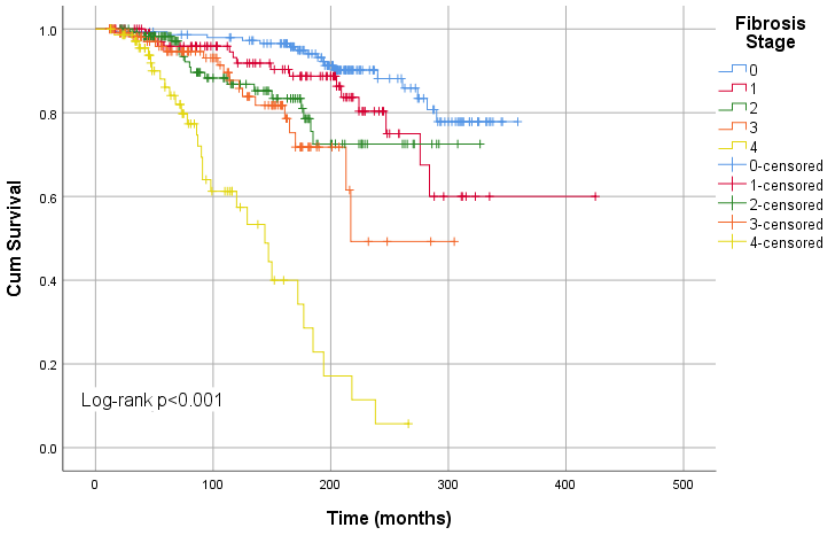
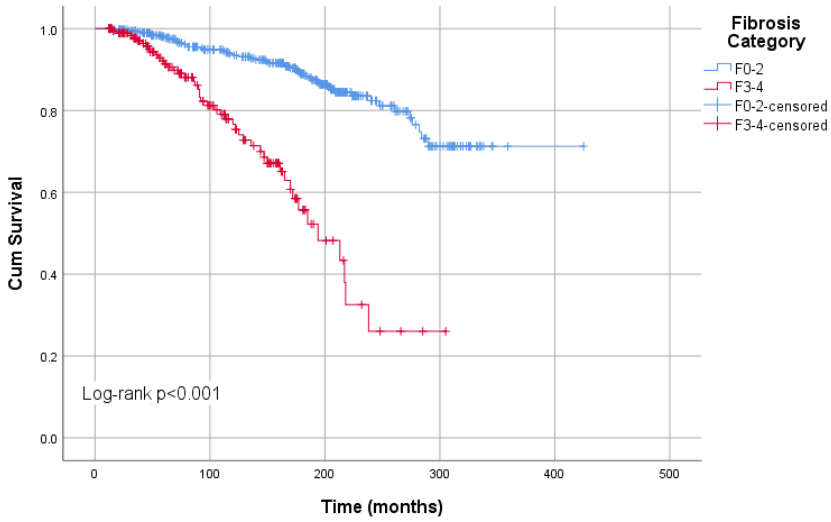


Figure 4.24. Effect of advanced fibrosis at baseline on Kaplan Meier Survival curves in all-cause mortality. From the curves below it can be seen that those with advanced fibrosis, defined as F3-4, have a higher risk of mortality than those with milder fibrosis (F0-2), p<0.001.



These histological variables were again explored for proportionality and advanced fibrosis (F3-4) was found to be proportional but fibrosis stage (i.e., F0-4) was not and therefore as discussed above the HRs for fibrosis stages may be over-estimated with time. Age and BMI were added into the models as control variables, but BMI was not found to be significant (p= 0.226 for Fibrosis Stage and p=0.165 for Advanced Fibrosis) and thus was removed. Once again sex was added as a stratum to each model and not found to have a significant influence on the outcomes as can be seen from figure 4.25 below.

Figure 4.25. Sex as a stratum in Cox Regression models for Fibrosis stage and Advanced fibrosis. The graphs below show that the addition of gender to cox regression models exploring these factps as predictors of mortality had no significant impact and therefore this was not used as a control in the models.

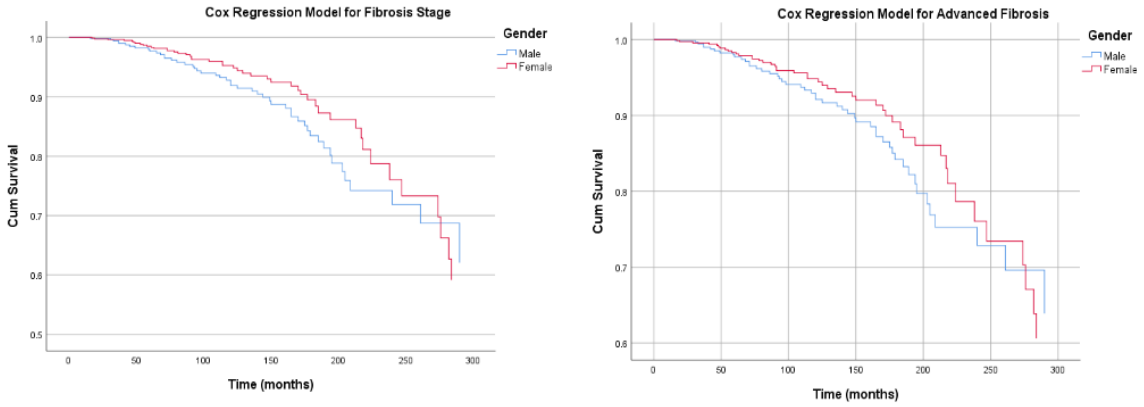


Table 4.16. Baseline histology that predict mortality by Cox Regression. Models were created exploring Fibrosis stage and the presence of Advanced fibrosis at baseline as predictors of mortality after these were found to be significant in univariate analysis. These factors were tested individually and then controlled for by age, and even after controls both were significantly associated with an increased risk of mortality in the Newcastle NAFLD cohort, p<0.001 for both. The table below demonstrates the hazard ratios generated from these models. BMI was not added as a control as this was not found to be significant and therefore removed from the model.

Variable	Log-rank p	HR (95%CI)	aHR (95%CI)*	p
Fibrosis • 0 (ref) • 1 • 2 • 3 • 4	<0.001	 1.89 (0.94 – 3.78) 2.85 (1.44 – 5.62) 4.21 (2.11 – 8.42) 16.49 (8.74 – 31.12)	 1.56 (0.77 – 3.15) 2.19 (1.11 – 4.33) 3.07 (1.52 – 6.20) 8.31 (4.31 – 16.01)	<0.001
Advanced Fibrosis	<0.001	4.75 (3.10 – 7.28)	3.36 (2.17 – 5.21)	<0.001
*Adjusted for Age.				

After controlling for age, Fibrosis stage remained an independent predictor of all-cause mortality with F4 present at baseline being associated with an aHR of 8.31 (95% CI 4.31 – 16.01). The presence of advanced fibrosis at baseline (F3-4) was also associated with a significant increased risk of mortality (p<0.001, aHR 3.36 95% CI 2.17 – 5.21).

4.9 Chapter Summary

The Newcastle NAFLD cohort were followed up for a mean period of 11.8 years and over the course of this time there was a significant increase in the prevalence of co-morbidities in the entire cohort including T2DM, IHD, CKD, Stroke and CCF. There were 112 deaths during the follow-up period with the mean time to death 10.1 years. The most common cause of death in the cohort was liver related (28.5%) followed by cardiovascular disease (20.5%) and malignancy (20.5%). Factors that may predict all-cause mortality were explored and there were various baseline characteristics identified as independent risks for all-cause mortality including age, the presence of T2DM or IHD and a FIB-4 or NFS score in the "indeterminate" or "high risk" category.

4.10 Discussion

The Newcastle NAFLD cohort reports on a large single centre, well characterised cohort of NAFLD patients with a follow-up period of over 10 years. This enables greater detail to be explored with regards the long-term outcomes of these patients. As described in chapter 1, the literature reports that the leading cause of death in patients with NAFLD is cardiovascular disease, followed by extrahepatic malignancies and then liver related death including decompensation and HCC. Within the Newcastle cohort 28.6% of the deaths were due to underlying liver disease which is in keeping with what was found by Stepanova *et al*[36] in a multicentre study of 289 patients with NAFLD in the USA. They demonstrated underlying liver disease was the cause of 26.1% of deaths in this study. However, there is a significant variation in the proportion of liver related deaths reported in long-term outcomes studies. Vilar-Gomez and colleagues[253] reported as high as 91.9% of deaths in their cohort were due to liver disease, while Sebastiani *et al*[254] documented 45.5%. These proportions are significantly higher than those reported by Ekstedt and colleagues in two long-term follow-up studies were 7.7-9% of deaths were due to liver disease.[93, 97] One of the key differences in these studies is Vilar-Gomez included only patients with F3-4 and Sebastiani's cohort also had over a third of patients with F3/4 at baseline, which would increase the likelihood of a liver related outcome. More recently in an interesting population-based study with over 10,000 participants and 4,000 with NAFLD, Simon *et al* demonstrated liver related mortality in 13.8% of deaths over a 14-year follow-up. This is more likely to be a representation of a general population mortality rate, and the Newcastle NAFLD cohort

whilst it does include patients of all fibrosis stages at baseline (F0-4) is likely to have a higher proportion of advanced disease than a UK population given the selection is from a specialist Hepatology centre.

The mean age of mortality in the Newcastle NAFLD cohort (64 years) is notably lower than the life expectancy of the local general Newcastle population (men 77.9 years, women 81.9 years).[339] This may be in part due to the patients who attend the tertiary specialist service NAFLD clinic come from across the North-East region where notably other areas have a lower life expectancy but is also likely a reflection of increased mortality with underlying NAFLD and co-morbidities seen in this cohort. Whilst the existing literature does not report mean age at death for comparison to other studies, an overall increased mortality in the NAFLD cohort compared to the general population is widely agreed upon; Ekstedt *et al* [97] reported an overall mortality HR 1.29, Hagstrom[98] HR 1.14 and Simon(252) aHR 1.93 when comparing to controls. The Newcastle NAFLD cohort is the first UK based study to demonstrate a significant reduction in life expectancy which highlights the pressing need for appropriate monitoring of these patients and the development of effective drug therapies.

One of the key aims of this chapter was to explore any factors that may be prognostic for all-cause mortality. There have been several factors identified in the literature, with Fibrosis stage being the key indicator of poorer outcomes.[97-99, 253, 254] Within the Newcastle NAFLD cohort it was also found that advanced fibrosis (F3/4) or cirrhosis was associated with a significantly increased risk of all-cause mortality (aHR 3.36 for advanced fibrosis and aHR 8.31 for F4). Sebastiani[254] similarly reported that the presence of advanced fibrosis (F3/4) was associated with aHR 3.14 for all-cause mortality. This study also demonstrated those patients with “high risk” FIB-4 and NFS scores also had an increased risk of mortality (aHR 6.33, aHR 11.9 for each score respectively) which was also apparent from the Newcastle NAFLD cohort. This is likely to be an important result in allowing for appropriate risk management of patients referred to NAFLD services.

Recent reports from the European Liver Transplant Registry (ELTR)[340] and the United Network for Organ Sharing (UNOS)[341] databases have shown that NAFLD has been the fastest growing indication for liver transplant over the last twenty years.[342] There has also been a sharp increase in the risk of HCC in NAFLD patients over this time period with one study indicating an increase in incidence from 2.1% to 16.2%[343], which is also becoming another leading indication for transplant in the NAFLD group. It is therefore interesting that

the rate of liver transplantation within the Newcastle NAFLD cohort is proportionally very low, only 1.7% received a liver transplant during the follow-up period. There are several reasons why this may be the case, but most likely the biggest factor contributing to this is the presence of multiple other co-morbidities in the NAFLD cohort. As can be seen from the Newcastle cohort there was a high prevalence of co-morbid conditions such as T2DM, IHD, CCF, CKD and stroke, by the final event which is likely to have had a significant impact on the eligibility for liver transplantation. Another factor likely to have affected the number of patients eligible for consideration of transplantation is age. NAFLD has been reported as a slowly progressive disease with a reported increase in fibrosis stage over 7-14 years,[45] depending on the presence of NASH, and the rate of progression from compensated to decompensated cirrhosis over a 2 year period has been reported at just 19%.[344] Whilst there is no official age limit in the U.K. for consideration of transplant it is likely with the slow rate of disease progression and decompensation that many patients did not reach the point of needing consideration for transplant until they were older, and perhaps no longer suitable for major surgery.

Cardiovascular disease is widely reported as one of the top causes for mortality in patients with NAFLD worldwide.[93, 99, 255] Within the Newcastle NAFLD cohort this is also true with 20% of the deaths due to cardiovascular disease and therefore it is unsurprising that the incidence of those diagnosed with IHD had increased significantly between the baseline and the final clinical visit of the study. Several studies have been undertaken to explore any factors that may predict the development of CVD or death due to heart disease and in chapter 3 it could be seen that there were several differences in characteristics between the IHD groups including older age and significantly higher mean non-invasive scores in those with IHD. The non-invasive scores have been shown to be prognostic of IHD outcomes in several studies. Chun and colleagues [345] demonstrated a higher FIB-4 result was associated with a HR 1.163 for the development of IHD, and Önnerhag *et al* identified that those with "high risk" FIB-4 results had a HR 6.52 for cardiovascular disease over a mean follow-up of 18.8 years.[346] More recently a UK based GP population study of over 40,000 patients also found a "high risk" FIB-4 score to be associated with an increased incidence of cardiovascular events.[347] It is therefore interesting to note that by the time of follow-up there was no longer a significant difference between the non-invasive scores, nor in the risk

categories when age adjusted cut-offs were applied, which may suggest age was the main factor predicting outcomes.

In summary, this chapter has reported the follow-up characteristics of the Newcastle NAFLD cohort and explored in detail those who died during the study. As documented throughout, the study has been limited by unavailable clinical details in a proportion of patients who were lost to follow-up, however there remained a significant number of patients with complete clinical details to enable a comprehensive description of this NAFLD cohort. This cohort was shown to have a significant increase in the incidence of various co-morbidities and clinical outcomes of interest, in particular T2DM and IHD, which have both been found to be independently prognostic for mortality, even after controlling for fibrosis stage. The non-invasive scores, FIB-4 and NFS, were also shown to prognosticate for mortality, which is in keeping with other studies and may provide supportive evidence for their use clinical management in this capacity. Liver-specific mortality outcomes and factors that may prognosticate for these will be explored in greater detail in the next chapter.

Chapter 5: Liver Mortality Outcomes

5.1 Introduction

Chapter 4 explored the all-cause mortality of those who died during the follow-up period of the study and demonstrated 28.6% of all deaths in the Newcastle NAFLD cohort were due to liver disease. This figure is in keeping with the current literature which suggests that liver disease (cirrhosis and HCC) is one of the top three causes of mortality in patients with NAFLD. In a large population study, Simon *et al* [252] found that cirrhosis and HCC accounted for 14.4% of mortality in the NAFLD patients, compared to 1.7% of an age and sex matched general population. While Vilar-Gomez *et al* [253] found in a cohort of NAFLD patients with "advanced fibrosis" (F3-4) 85% of deaths were liver related. Studies have generally reported that liver disease is the cause of death in 7-35% of a general NAFLD cohort. [32, 36, 97-99, 254, 257]

These studies have explored factors which may predict liver mortality. Fibrosis stage has consistently been the strongest histological feature that independently predicts outcomes.[97-99, 253] Other clinical features that have been shown to contribute to liver mortality include the presence of T2DM, increasing age, low albumin levels and low platelet count, the latter two indicating advanced fibrosis. However, the natural history of NAFLD remains poorly understood and, given the increasing prevalence of NAFLD globally, a better understanding of other clinical factors that predict an increased risk of a liver related death is of the utmost importance to inform practice.

5.2 Aims

This chapter will:

1. Describe the characteristics of those who died of liver disease during the follow-up period.
2. Explore any factors associated with liver mortality and describe the relationships.
3. Examine the use of non-invasive scores to predict death by liver disease in a NAFLD cohort.

5.3 Liver Mortality Definition

Liver Mortality for this study is defined as cause of death documented in section I (a, b or c) of the death certificate containing any diagnosis pertaining to their underlying NAFLD. This included terms such as decompensated liver cirrhosis, NASH cirrhosis, chronic liver disease and Hepatocellular Carcinoma. This indicates the clinician completing the death certificate felt that the patient died as a direct result of their underlying NAFLD diagnosis.

This section will explore the characteristics of all patients who died as a result of their liver disease as defined by death certificate documentation.

It is generally acknowledged in the existing literature that liver transplantation is classified as a surrogate for death as the mortality in this group of patients who do not receive a liver transplant is reported as high as 50% in 2 years [348]. This chapter will therefore include those who underwent liver transplantation during the follow-up period in the analysis of the liver mortality group.

5.4. Characteristics of liver mortality group

5.4.1 Baseline

Forty two patients met the criteria for liver mortality during the follow-up period, 32 died as a result of their underlying NAFLD and 10 underwent a liver transplant. This accounts for 6.9% of the entire cohort. The mean age of death in this group was 70 ± 11 years and the median time to death or transplant was 8.4 years (1.3-20.6 years) from the initial clinical visit of the study.

The baseline characteristics of this group are displayed in table 5.1 below. The mean age at baseline was 60 years and 54.8% were male. Liver blood test results at the initial clinical visit showed elevated ALT, AST and GGT but normal synthetic function (normal albumin, platelet, and PT levels). A high proportion of this group had associated co-morbidities. T2DM was diagnosed in 65.9%, 72.7% were known to have HTN and of note 45.0% already had a diagnosis of IHD by the initial clinical visit. As may be expected 81.0% of the group already had a diagnosis of cirrhosis at baseline.

Table 5.1. Baseline characteristics of those who died from liver related mortality in the Newcastle NAFLD cohort. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range. Results were rounded to decimal or integer numbers as would be used clinically.

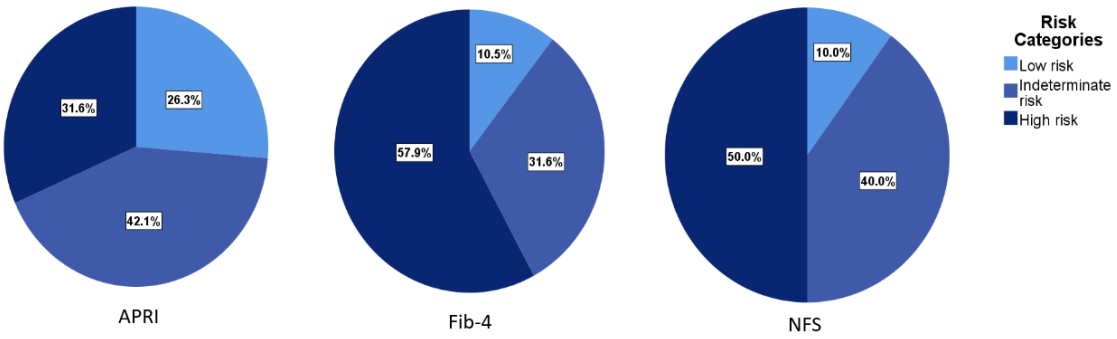
Variable	n	Result
Age (years)	42	60 ± 9
Sex (male)	42	23 (54.8%)
BMI (kg/m^2)	36	34.0 (25.7-50.9)
ALT (0 – 40) [U/l)	40	50 (13-278)
AST (0 – 40) [U/l)	38	49 (20-218)
GGT (0 – 70) [U/l)	33	173 (56-2062)
Albumin (35 – 50) [g/l]	40	39 ± 5
Platelets (150 – 450) [x10^9/l]	40	168 ± 80
PT (10 – 13) [seconds]	28	13 (11-18)
IgA (0.8 – 3.0) [g/l]	37	4.86 ± 2.27
T2DM	41	27 (65.9%)
HTN	22	16 (72.7%)
MetS	24	17 (70.8%)
IHD	20	9 (45.0%)
Malignancy	24	3 (12.5%)
Cirrhosis	42	34 (81.0%)

The baseline results of the non-invasive scoring tools were explored for this group and the results are shown in table 5.2 below. Given 80% of these patients were already known to be cirrhotic at baseline it is unsurprising that each of these scores mean results are indicative of advanced fibrosis and the risk category distribution, shown in figure 5.1 below, is also in keeping with a high proportion of “high risk” patients. This is also demonstrated in figures 4.1-4.3 in the previous chapter, which shows the movement of individuals between risk categories over the course of the follow-up.

Table 5.2. Results of the baseline non-invasive tool scores of those who died a liver related death.

Non-invasive Tool	n	Result
AST/ALT Ratio	38	1.00 (0.49-2.54)
APRI	38	0.83 (0.20-3.97)
FIB-4 Score	38	3.00 (0.93-12.75)
NFS	30	0.92 ± 1.53
Child's Pugh Score	34	6 ± 1

Figure 5.1. Proportion of patients in each risk category of non-invasive tool at baseline in liver mortality patients. Cut offs used were APRI 0.5 and 1.5, FIB-4 1.30 and 2.67, and NFS -1.455 and 0.676.



Of the 34 patients with cirrhosis at baseline 58.8% (20) of these were diagnosed by histology, 38.2% (13) had clinical signs of cirrhosis and 2.9% (1) was radiologically diagnosed. The result of the histology from baseline biopsies in the liver mortality group are displayed in table 5.3 below. Of note only 18 patients were characterised as F4 using the Kleiner classification, but a further 2 patients was reported as F5 on extended fibrosis reporting and therefore classified as cirrhotic. As may be expected, a high proportion of patients were found to have NASH (75.0%) at baseline and a further 60.7% met the criteria for Fibrosing Steatohepatitis on biopsy.

Table 5.3. Baseline histological features, as defined by the NASH CRN criteria, of those who died of a liver related death. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

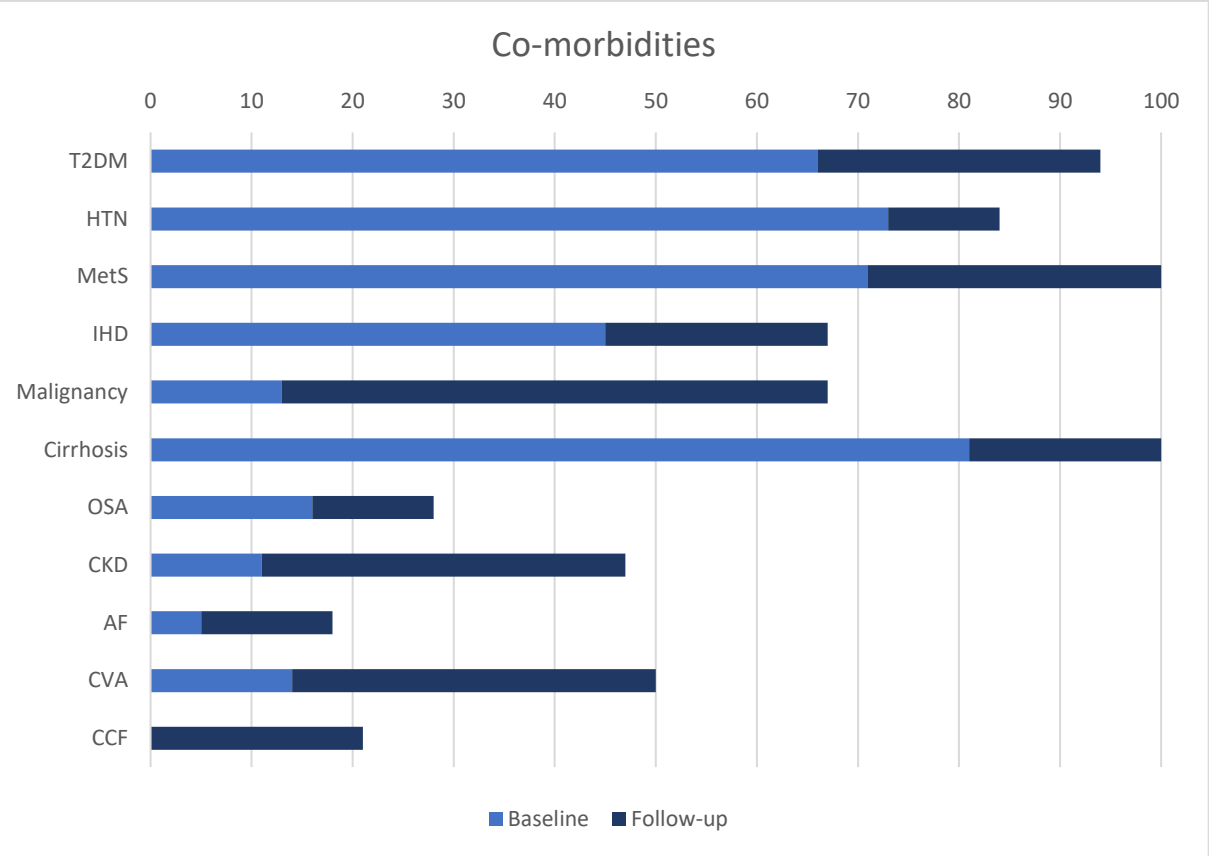
Variable	n	Result
Steatosis • 0 • 1 • 2 • 3	28	 2 (7.1%) 9 (32.1%) 13 (46.4%) 4 (14.3%)
Ballooning • 0 • 1 • 2	28	 4 (14.3%) 15 (53.6%) 9 (32.1%)
Inflammation • 0 • 1 • 2 • 3	28	 5 (17.9%) 14 (50.0%) 9 (32.1%) 0
Fibrosis • F0 • F1 • F2 • F3 • F4	28	 1 (3.6%) 2 (7.1%) 2 (7.1%) 5 (17.9%) 18 (64.3%)
Fibrosis category • No significant (0-1) • Clinically significant (2-4) • Advanced (3-4)	28	 3 (10.7%) 25 (89.3%) 23 (82.1%)
NASH (present)	28	21 (75.0%)
NAS Score Categories • 1-2 • 3-4 • ≥5	28	 5 (17.9%) 13 (46.4%) 10 (35.7%)
SAF Activity Score	28	2 ± 1
Fibrosing steatohepatitis	28	17 (60.7%)

5.4.2. Final event.

As previously acknowledged in chapter 4, incomplete clinical data were available for a number of patients who died and were no longer under the care of the Newcastle Hospitals and as a result the overall picture of these patients' health may be incompletely defined. From the data that were available there was an overall increase in the prevalence of co-

morbidities and clinical outcomes of interest. Figure 5.2 below shows the percentage of patients in the liver mortality group that had been diagnosed with co-morbidities such as T2DM and HTN and those who developed clinical outcomes such as CKD, stroke and IHD by the time of death. This excludes those with missing data for each individual diagnosis.

Figure 5.2. Prevalence of co-morbidities in the liver mortality group at time of death.



The proportion of these co-morbidities had increased in the entire cohort by the end of the study, and these were notably more prevalent than in those who were still alive at the end of follow up [T2DM (93.9% vs 69.0%, p=0.002), IHD (66.7% vs 20.9%, p<0.001) and CKD (47.1% vs 13.9%, p=0.002)].

5.5. Comparison of baseline characteristics between alive vs liver-death groups.

The baseline characteristics of those who died a liver related death were compared to those who were still alive at the end of the study. The analysis excludes the group of patients who died during follow-up of an alternative cause of death, for example those who died of cardiovascular disease or non-HCC malignancy, as they were further explored in chapter 4.

5.5.1. Clinical demographics and co-morbidities.

Those who died were significantly older, 60 vs 52 years ($p<0.001$) and 54.8% were male, with no significant difference in sex ($p=0.627$) or BMI ($p=0.656$) seen between the groups. Baseline differences in liver related blood results indicated more advanced liver disease in those who died a liver-related death: lower ALT ($p=0.003$), albumin ($p<0.001$) and platelets count ($p<0.001$). Of interest there were also significantly higher IgA ($p<0.001$) and GGT ($p=<0.001$) levels in this group. Table 5.4 below documents the comparison of baseline characteristics of both groups.

Increased prevalence of co-morbidities and clinical outcomes were seen in the liver mortality group compared to those alive in T2DM (65.9% vs 44.4%, $p<0.001$), HTN (72.7% vs 50.2%, $p=0.002$) and IHD ($p<0.001$) and cirrhosis ($p<0.001$).

Table 5.4. Comparison of baseline characteristics between alive vs liver mortality groups. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Alive n= 488	Dead n= 42	p value
Duration of follow-up (months)	152 (12-425)	101 (16-247)	<0.002^{+}
Age (years)	52 (18-82)	60 ± 9	<0.001^{+}
BMI (kg/m^2)	34.0 (22.7-59.1)	34.0 (25.7-50.9)	0.656^{+}
Sex (male)	287 (58.8%)	23 (54.8%)	0.627^{+}
ALT (0 – 40) [U/l)	63 (10-355)	50 (13-278)	0.003^{+}
AST (0 – 40) [U/l)	43 (13-251)	49 (20-218)	0.126^{+}
GGT (0 – 70) [U/l)	78 (14-1141)	173 (56-2062)	<0.001^{+}
Albumin (35 – 50) [g/l]	45 ± 3	39 ± 5	<0.001^{+}
Platelets (150 – 450) [x10^9/l]	246 ± 71	168 ± 80	<0.001^{+}
PT (10 – 13) [seconds]	12 (9-19)	13 (11-18)	<0.001^{+}
IgA (0.8 – 3.0) [g/l]	2.61 (0.42-20.90)	4.86 ± 2.27	<0.001^{+}
AST/ALT Ratio	0.71 (0.22-2.91)	1.00 (0.49-2.54)	<0.001^{+}
APRI	0.47 (0.08-3.18)	0.83 (0.20-3.97)	<0.001^{+}
FIB-4 Score	1.16 (0.15-7.43)	3.00 (0.93-12.75)	<0.001^{+}
NFS	-1.41 ± 1.72	0.92 ± 1.53	<0.001^{+}
Child's Pugh Score	5 ± 0	6 ± 1	<0.001^{+}
T2 DM	216 (44.4%)	27 (65.9%)	0.009*
HTN	227 (50.2%)	16 (72.7%)	0.049*
MetS	273 (73.8%)	17 (70.8%)	0.812*
IHD	48 (12.2%)	9 (45.0%)	<0.001$^{\#}$
Malignancy	23 (5.1%)	3 (12.5%)	0.139$^{\#}$
Cirrhosis	62 (12.7%)	34 (81.0%)	<0.001*
$^{+}$Mann Whitney U, *Chi Square, #Fisher's Exact			

5.5.2. Non-invasive scoring tools.

Given the non-invasive tools were developed to predict advanced fibrosis, which has long been shown to be a predictor of liver mortality, it is unsurprising that there were significant differences in the results of these scores between the alive and the liver mortality groups.

The mean result for each score was significantly higher in the liver mortality group as seen in table 5.4 above (APRI p<0.001, Fib4 p<0.001, NFS p<0.001). Figures 5.3 - 5.5 below demonstrate the outcomes of liver mortality vs alive at follow-up based upon the baseline non-invasive risk category of each score.

Figure 5.3. Sankey diagram demonstrating survival at the end of the study stratified by APRI risk category at baseline. Cut-offs used were <0.5 "low risk", 0.5-1.49 "indeterminate risk" and >1.5 "high risk" and it can be seen that fewer "low risk" patients at baseline had died compared to indeterminate or high risk.

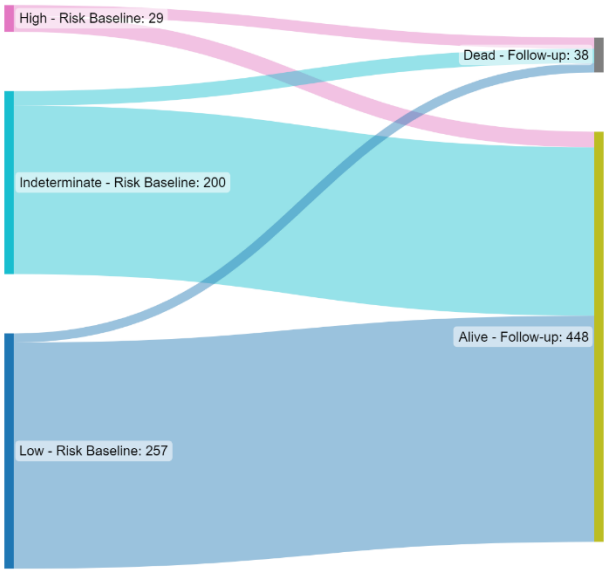


Figure 5.4. Sankey diagram demonstrating survival at the end of the study stratified by FIB-4 risk category at baseline. Cut-offs used were <1.30 "low risk", 1.30-2.67 "indeterminate risk" and >2.67 "high risk" and it can clearly be seen that a higher proportion of those "high risk" at baseline died compared to those "low risk".

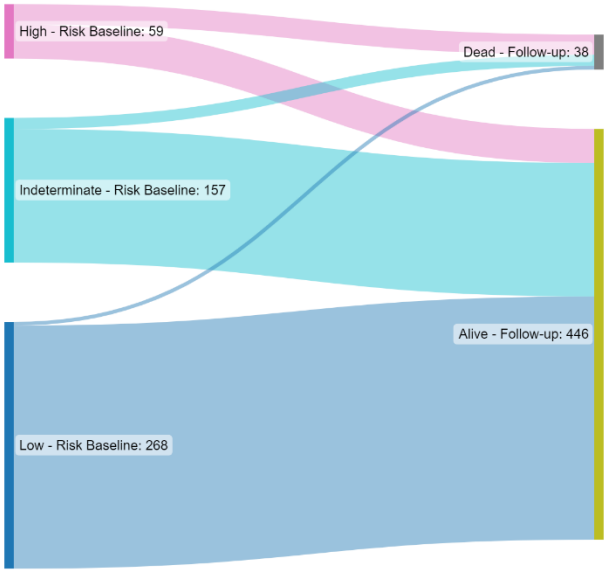
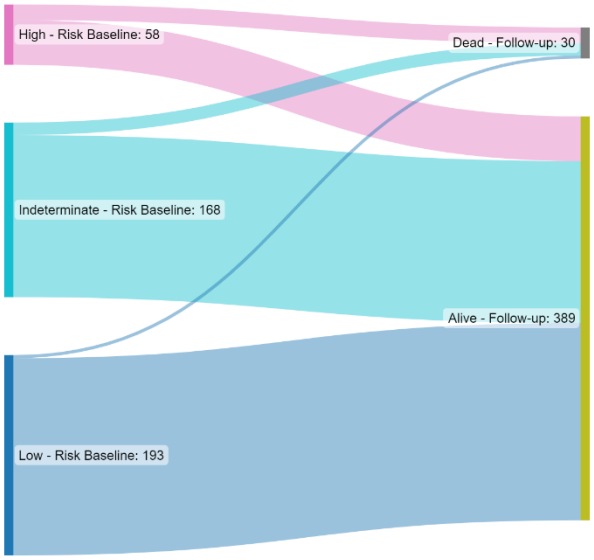


Figure 5.5. Sankey diagram demonstrating survival at the end of the study stratified by NFS risk category at baseline. Cut-offs used were <-1.455 "low risk", -1.455-0.676 "indeterminate risk" and >0.676 "high risk" and from the graph below it can be seen that a higher proportion of those "high risk" at baseline died during the follow-up compared to those "low risk".



5.5.3. Histology.

The baseline histology results of those who were alive were compared to the liver mortality group, shown below in table 5.5. As may be expected there was a significant difference in fibrosis stage between the groups ($p<0.001$), but of interest the liver mortality group had higher ballooning scores ($p=0.026$). There was no significant difference in the presence of NASH (75.0% vs 62.2%, $p=0.228$) or Fibrosing Steatohepatitis (60.7% vs 45.3%, $p=0.122$) between the groups seen.

Table 5.5. Comparison of baseline histology, as defined by NASH CRN criteria, between patients alive at the end of the study vs those who died a liver death. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	Alive	Dead	p value
Steatosis			0.115*
• 0	1 (0.2%)	2 (7.1%)	
• 1	143 (29.9%)	9 (32.1%)	
• 2	215 (44.9%)	13 (46.4%)	
• 3	120 (25.1%)	4 (14.3%)	
Ballooning			0.026*
• 0	166 (34.7%)	4 (14.3%)	
• 1	212 (44.3%)	15 (53.6%)	
• 2	101 (21.1%)	9 (32.1%)	
Inflammation			0.690*
• 0	113 (23.6%)	5 (17.9%)	
• 1	218 (45.5%)	14 (50.0%)	
• 2	137 (28.6%)	9 (32.1%)	
• 3	11 (2.3%)	0	
Fibrosis			<0.001*
• 0	133 (27.8%)	1 (3.6%)	
• 1	96 (20.0%)	2 (7.1%)	
• 2	107 (22.3%)	2 (7.1%)	
• 3	100 (20.9%)	5 (17.9%)	
• 4	43 (9.0%)	18 (64.3%)	
Fibrosis category			
• No significant	229 (47.8%)	3 (10.7%)	<0.001*
• Clinically significant	250 (52.2%)	25 (89.3%)	
• Advanced	143 (29.9%)	23 (82.1%)	<0.001*
NASH	300 (62.6%)	21 (75.0%)	0.228+
NAS Score Category			0.888*
• 1-2	122 (25.5%)	5 (17.9%)	
• 3-4	166 (34.7%)	13 (46.4%)	
• ≥5	191 (39.9%)	10 (35.7%)	
SAF Score	2 ± 1	2 ± 1	0.128*
Fibrosing steatohepatitis	217 (45.3%)	17 (60.7%)	0.122+
*Kruskal Wallis, +Chi Square			

5.6. Predictors of liver mortality.

Building from the univariate analysis of factors above, Kaplan Meier survival curves were generated, and multivariate Cox regression was then undertaken to further examine any

variables at baseline that may be able to predict liver mortality. Any variable that met the univariate significance of p≤0.100 was considered for this analysis, which was again divided into categories as in chapter 4: blood results, non-invasive scoring tools, clinical outcomes, and histology, to mitigate for possible collinearity of variables. This section continues to exclude the 74 patients that died during the follow-up period of a non-liver related death.

5.6.1. Liver blood tests and clinical parameters

The routinely used liver blood tests which had been significant in univariate analysis were forced into a backwards stepwise Cox regression model which therefore included the following variables: Age, ALT, GGT, Albumin, Platelets, PT, and IgA.

This regression model identified two variables which were not significant in controlling for other variables in the model and these were therefore removed, ALT (p=0.933) and PT (p=0.845). The model was then re-run with the remaining five variables, and IgA was no longer found to be significant (p=0.260). This final variable was removed, and the updated model demonstrated that the remaining clinical factors were all found to be independent predictors of liver mortality: Age, GGT, Albumin and Platelet count. The results of this model and the hazard ratios for each variable are shown in table 5.6 below.

Table 5.6. Baseline blood tests that predict of liver mortality by multivariate Cox regression modelling. Factors significant in univariate analysis (age, ALT, GGT, Albumin, Platelets, PT and IgA) were included in backwards stepwise Cox regression models. ALT, PT and IgA were not found to be significant, but the others were found to be independent predictors of liver mortality in the Newcastle NAFLD cohort. The table below displays the hazard ratios generated for each variable.

Variable	aHR (95% CI)	p
Age	1.07 (1.03, 1.11)	0.001*
GGT	1.002 (1.00, 1.00)	<0.001*
Albumin	0.69 (0.610, 0.77)	<0.001*
Plt	0.989 (0.98, 0.996)	0.003*
*Multivariate Cox Regression		

As was seen in all-cause mortality analysis both Albumin and Platelet count have a "protective" hazard ratio (<1.00) likely due to the known pattern of both these blood tests falling in more advanced liver disease. GGT has also been shown to be a good predictor of

liver mortality(108), albeit not disease specific, and this was also found to be an independent predictor in the Newcastle NAFLD cohort.

5.6.2. Non-invasive scoring tools.

The non-invasive tools were then analysed using Kaplan Meier log-rank test, multivariate Cox regression modelling to examine their potential to predict liver mortality. As previously highlighted in chapter 4, these tools contain multiple shared elements in their formulations, and it was therefore not possible to enter each tool into a single model due to collinearity.

Each non-invasive tool's categorical results (i.e., low, indeterminate, and high risk) were analysed by log-rank tests and Kaplan Meier survival curves were generated. As was found when analysing all-cause mortality, there was a significantly higher mortality in the high-risk groups of each tool ($p<0.001$ for all). Figures 5.6 – 5.8 below demonstrate these survival curves and while a clear difference in cumulative survival can be seen in each risk category for each tool, there is perhaps a more notable split between the indeterminate and high-risk groups in the Fib4 and NFS tools suggesting they have greater accuracy in predicting mortality between categories.

Figure 5.6. Kaplan Meier survival curves of liver related mortality stratified by APRI risk categories at baseline. Cut-offs used were <0.5 "low risk", 0.5-1.49 "indeterminate risk" and >1.5 "high risk". From the graph it can be seen that those in the "high-risk" group had an increased risk of mortality (p<0.001) and shorter survival time than those in the "low-risk" group.

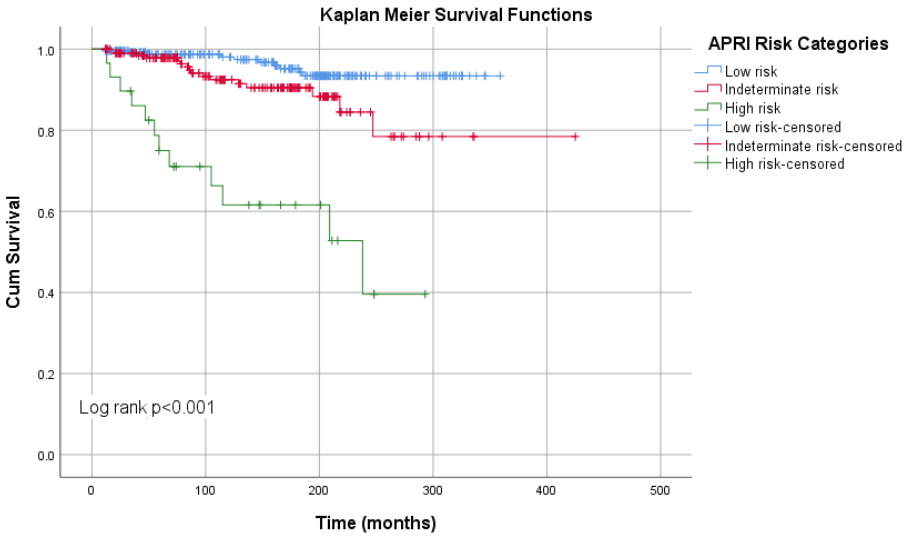


Figure 5.7. Kaplan Meier survival curves of liver related mortality stratified by of FIB-4 risk categories at baseline. Cut-offs used were <1.30 "low risk", 1.30-2.67 "indeterminate risk" and >2.67 "high risk". From the graph it can be seen that those in the "high-risk" group had a significantly increased risk of mortality (p<0.001) and shorter survival time than those in the "low-risk" group.

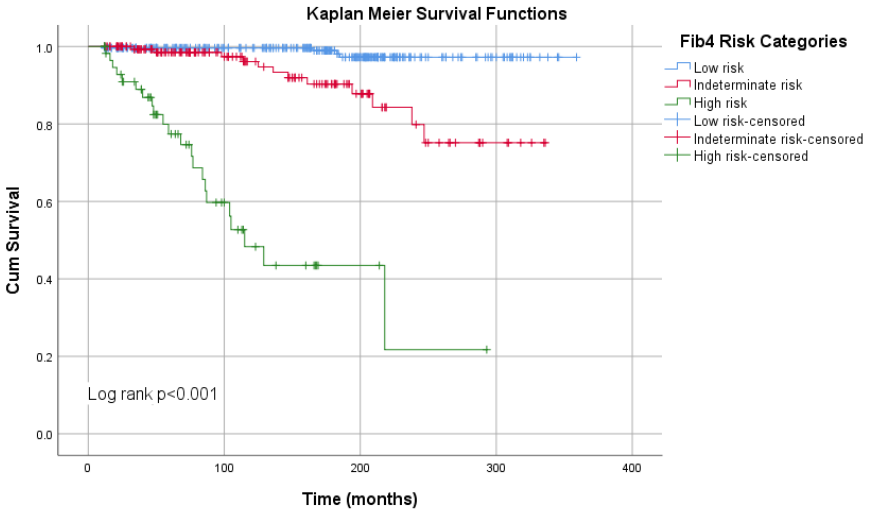
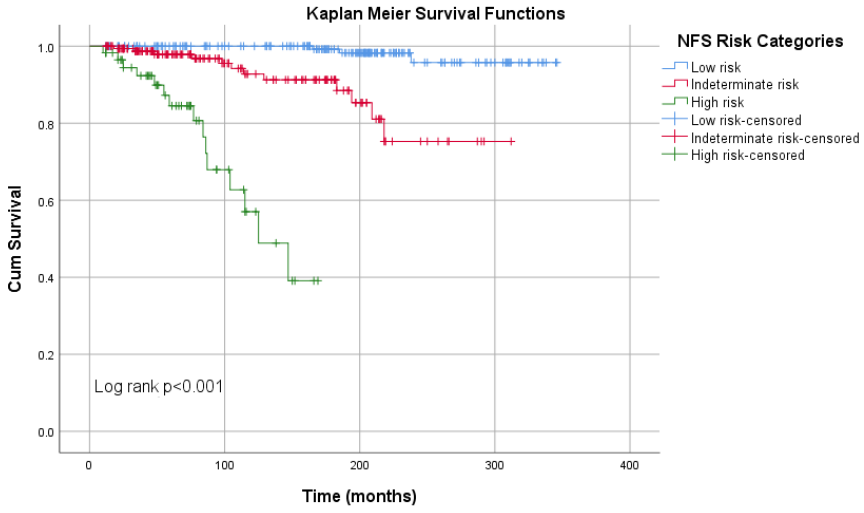


Figure 5.8. Kaplan Meier survival curves of liver related mortality stratified by of NFS categories at baseline. Cut-offs used were <-1.455 "low risk", -1.455-0.676 "indeterminate risk" and >0.676 "high risk". From the graph it can be seen that those in the "high-risk" group had a significantly increased risk of mortality (p<0.001) and shorter survival time than those in the "low-risk" group.



As in chapter 4, hazard ratios for each tool were then produced by univariate Cox Regression modelling examining both the mean score results and the risk categories. The results for the risk categories refer to comparison with "low risk" as the reference point. Unlike in all-cause mortality the risk category variables were found to be "proportional" and therefore could be used in the model without risk of overestimated risk with time. Each of the tools demonstrate an increased risk of liver mortality, with a patient being identified as "high risk" from the FIB-4 score being associated with a hazard ratio of 65.59 (95% CI 22.07, 194.96, p<0.001). Table 5.7 below shows the results of the hazard ratios for each of the tools.

Table 5.7. Cox Regression hazard ratios of baseline non-invasive tools predicting all-cause mortality. Using "low-risk" as the comparator reference for each score, and the cut-offs as described throughout (APRI <0.5, 0.5-1.5, >1.5. FIB-4 <1.30, 1.30-2.67, >2.67. NFS <-1.455, -1.455-0.676, >0.676.), the table below again shows patients with "high-risk" scores at baseline have an increased risk of liver mortality and the hazard ratios associated.

Variable	HR (95% CI) -score means	p	HR (95% CI) * -categories	p
AST/ALT Ratio	9.36 (5.42, 16.17)	<0.001	-	-
APRI • Low (ref) • Indeterminate • High	3.46 (2.42, 4.94)	<0.001	 Reference 2.45 (1.11, 5.42) 12.66 (5.46, 29.34)	 0.027 <0.001
FIB-4 Score • Low (ref) • Indeterminate • High	1.99 (1.74, 2.28)	<0.001	 Reference 6.84 (2.20, 21.26) 65.59 (22.07, 194.96)	 0.001 <0.001
NFS • Low (ref) • Indeterminate • High	3.01 (2.33, 3.88)	<0.001	 Reference 10.39 (2.89, 37.29) 99.19 (24.87, 395.56)	 <0.001 <0.001
Child Pugh	2.35 (1.79, 3.08)	<0.001	-	-
*Using "low risk" as the reference for the cox regression model				

From the Kaplan Meier survival curves and the hazard ratios for each of these scores it can clearly be seen that those who fall into the "high-risk" category have a significantly increased risk of a liver related death compared to those who were in the "low-risk" category.

5.6.3. Co-morbidities and Clinical Outcomes of Interest

Further exploration of the co-morbidities and clinical outcomes of interest present at the time of the baseline visit that were significant in univariate analysis was also undertaken. As BMI and sex had not been found to be significantly different between those alive and those who died of liver disease at follow-up, age was the only factor that was used as a control when undertaking Cox regression analysis. Kaplan Meier survival curves and log rank tests were also performed for each factor.

Figures 5.9 and 5.10 below show the effect of T2DM and HTN on survival. As with all-cause mortality, those with each co-morbidity can be seen to have an increased liver mortality over time, p<0.001 and p=0.002 respectively.

Figure 5.9. Effect of baseline T2DM on Kaplan Meier survival curves in liver related mortality. The curves demonstrate that patients with a diagnosis of T2DM at baseline are associated with an increased risk of liver death and shortened survival time than those without diabetes (p<0.001).

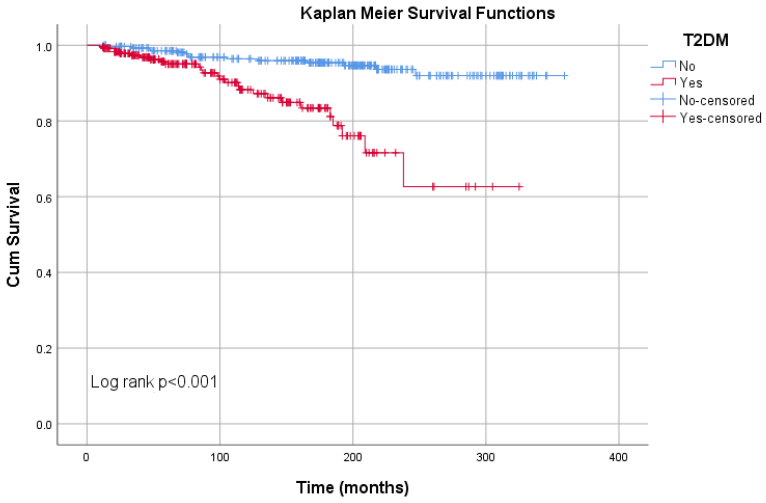
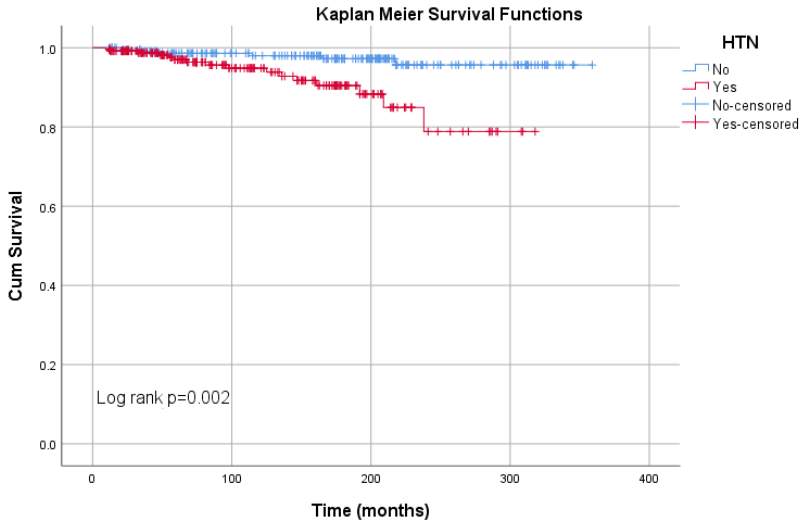


Figure 5.10. Effect of baseline HTN on Kaplan Meier survival curves in liver specific mortality. The curves demonstrate that patients with a diagnosis of HTN at baseline are associated with an increased risk of liver death (p=0.002) and shortened survival time than those without.



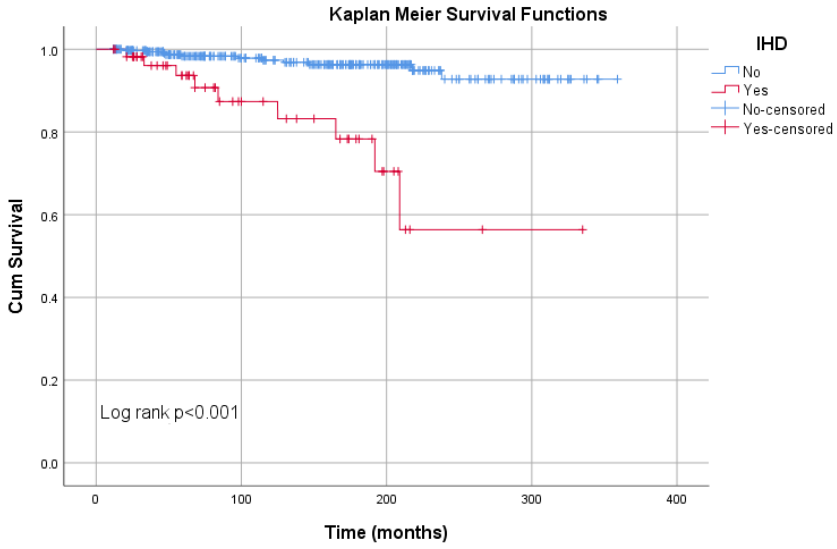
These factors were then further assessed by Cox regression to control for age. Both variables were again reviewed for proportionality before being used in a model and HTN remained "non-proportional", raising the possibility of over-estimated risk with time. However, T2DM was found to be proportional and therefore hazard ratios can be considered fully accurate. After adjusting for age, the presence of T2DM was found to have a greater than 2-fold increased risk of liver mortality (aHR 2.50, p=0.011) but HTN was not found to meet significance (p=0.097), although did show a trend towards increased risk of mortality as can be seen in table 5.8 below.

Table 5.8. Baseline co-morbidities that predict mortality by Cox Regression. Models were created exploring T2DM and HTN as predictors of liver mortality. Both variables were controlled for age in the model and T2DM was found to have a greater than 2-fold increased risk of liver mortality (p=0.011), however HTN was not found to be significant (p=0.097). Hazard ratios generated from these models are displayed in the table below.

Variable	KM log rank p	HR (95%CI)	aHR (95%CI)*	p
T2DM	<0.001	4.46 (2.28, 8.69)	2.50 (1.24, 5.05)	0.011
HTN	0.002	4.07 (1.57, 10.54)	2.26 (0.86, 5.94)	0.097
*Adjusted for Age.				

Kaplan Meier survival curves and log rank tests were also performed on IHD, a clinical outcome of interest which was significant in univariate analysis. Cirrhosis was also found to be significant in univariate analysis, but this will be explored in the histology section below when examining Fibrosis. Figure 5.11 below demonstrates the survival curves stratified by the presence of IHD and there can clearly be seen to be an increase in liver mortality in the group of patients diagnosed with IHD at baseline, p<0.001.

Figure 5.11. Effect of a baseline diagnosis of IHD on Kaplan Meier survival curves in liver related mortality. The curves below show the presence of IHD was associated with an increased risk of a liver related death (p<0.001).



IHD was assessed for proportionality prior to generating Cox proportional hazard models and was found to be "proportional", allowing the inclusion in a model with no risk of overestimation of their effect with time. After controlling for age, a diagnosis of IHD remained an independent predictor of mortality, p=0.004. Table 5.9 below shows the hazard and adjusted hazard ratios for IHD.

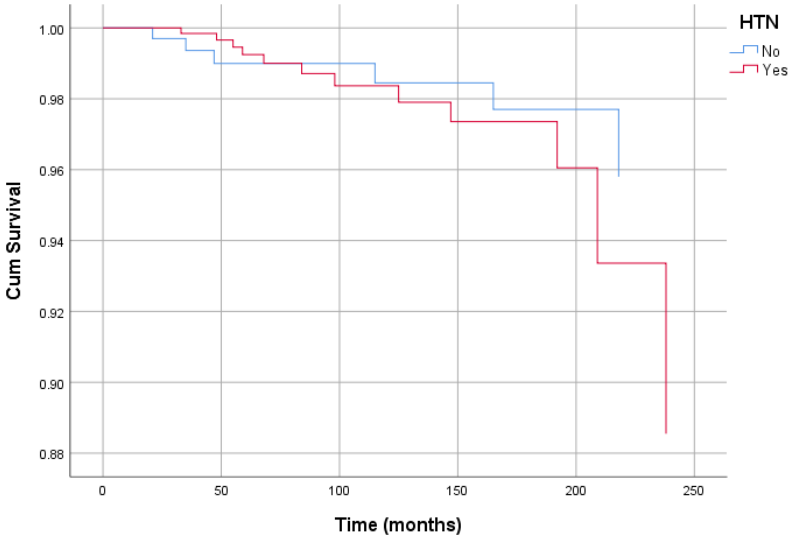
Table 5.9. Baseline clinical outcomes that predict mortality by Cox Regression. Models were created exploring IHD as a predictors of mortality. IHD was added to a model and then controlled for age. After controls the presence of baseline IHD was significantly associated with an increased risk of liver related mortality in the Newcastle NAFLD cohort, p=0.004. The table below demonstrates the hazard ratios generated from these models.

Variable	KM log-rank p	HR (95%CI)	aHR (95%CI)*	p
IHD	<0.001	7.43 (3.03, 18.25)	3.86 (1.53, 9.74)	0.004
*Adjusted for Age				

As T2DM was found to be proportional for this cohort (alive vs liver mortality), this was also added to the model to control for any confounding affect diabetes may have had on IHD but was found not to be significant (p=0.115).

HTN could not be added as a covariate as it was found to be "non-proportional", and therefore it was added as a stratum to the model with IHD and age. Figure 5.12 below shows the survival function of the model with HTN as a stratum, demonstrating no significant difference between the HTN groups.

Figure 5.12. Effect of HTN as a stratum in Cox regression model for IHD as a predictor of liver mortality. The presence of HTN could not be controlled for in the IHD model by adding the variable as a co-variate as it was "non-proportional", and therefore it was added as a stratum to test for any control it may add to the model. The graph below demonstrates there was no significant impact on the IHD model by the addition of HTN.



5.6.4. Histology

Finally, features of baseline histology were examined to explore their ability to independently predict liver mortality. As with the other factors, this was undertaken through Kaplan Meier survival curves and log-rank tests and multivariate Cox regression modelling. Only variables found to be significant (p<0.100) on univariate analysis were included: ballooning score, fibrosis stage and fibrosis categories. Fibrosis categories were simplified to explore the effects of advanced fibrosis (F0-2 vs F3-4).

When undertaking Kaplan Meier analysis, the generated log-rank p value is reflective of overall comparison for each factor. For example, in the survival curve for Ballooning (figure 5.13 below) it can be seen that there is a significant difference in survival between those who score 0 vs 2 (p<0.001) but comparing 1 vs 2 is not significant (p=0.068).

As expected, a fibrosis score of F4 at baseline demonstrated the most significant impact on survival over time (p<0.001) as can be seen in figure 5.14 below. A higher ballooning score at baseline also showed a clear trend of increasing mortality over time (p<0.001), as can be seen in the Kaplan Meier survival curves below.

Figure 5.13 Effect of baseline ballooning score on Kaplan Meier Survival curves in liver mortality. From the curves it can be seen that a higher degree of ballooning grade was associated with an increased risk of liver mortality over time (p<0.001).

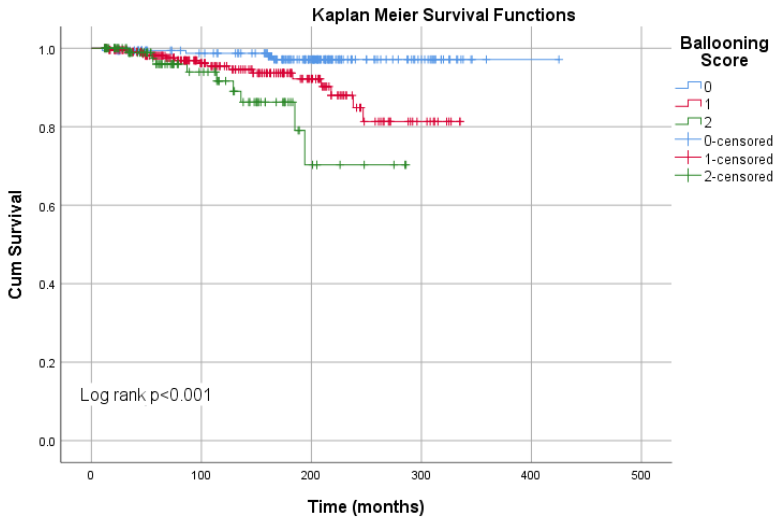


Figure 5.14 Effect of baseline Fibrosis on Kaplan Meier survival plots in liver mortality. The curves below clearly demonstrate a significantly increased risk of liver mortality and shortened survival time in patients with F4 at baseline (p<0.001).

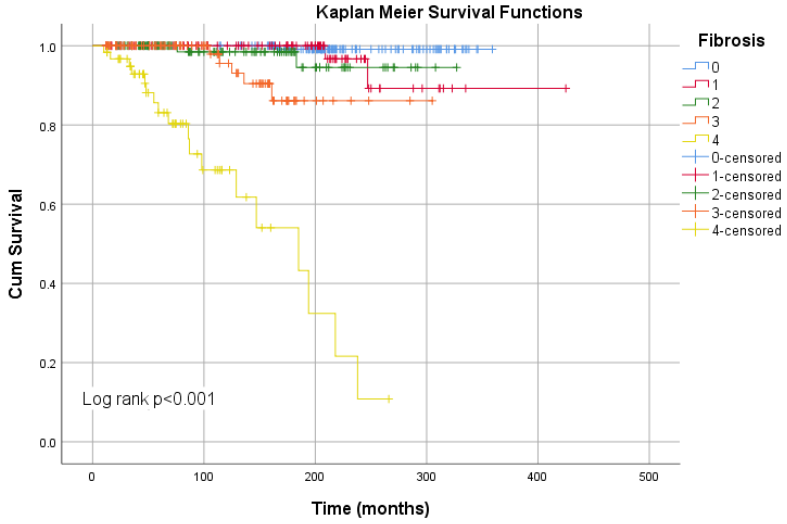
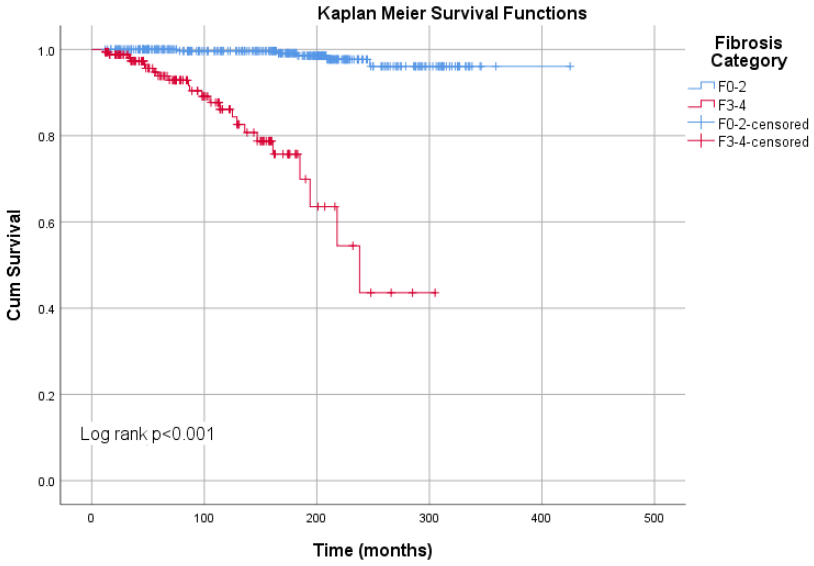


Figure 5.15 Effect of baseline fibrosis categories on Kaplan Meier survival curves in liver mortality. The presence of advanced fibrosis (F3-4) can clearly be seen to have a significant association with liver death (p<0.001).



Hazard ratios for these histological components were then generated from univariate Cox regression before adjusted hazards were calculated using age as a control. Of note only Fibrosis and Fibrosis categories were found to be “proportional” and therefore the effect of the estimated risk of each other factor may be exaggerated with time.

After adjusting for age, the presence of F4 fibrosis at baseline was the strongest predictor of liver mortality with an adjusted hazard ratio of 96.98 (95% CI 12.44, 756.29). Table 5.10 below displays the HR and aHRs for the histology components in predicting liver mortality.

Table 5.10. Baseline histological features that predict liver mortality by Cox Regression. Models were created exploring variable found significant on univariate analysis, Ballooning grade, Fibrosis stage and Advanced fibrosis. Using ballooning 0 and fibrosis stage 0 as the reference, these variables were added to the model and then in a second analysis controlled for by age and as can be seen from the table below Ballooning score 2, F3, F4 and Advanced fibrosis were found to be independent predictors of liver mortality (p=0.004, p=0.010, p<0.001 and p<0.001 respectively. The table below displays the hazard ratios for each histological feature.

Variable	Log-rank p	HR (95%CI)	aHR (95%CI)*	p
Ballooning • 0 (ref) • 1 • 2	<0.001~	 4.29 (1.42, 12.98) 8.91 (2.69, 29.59)	 3.10 (1.02, 9.45) 5.96 (1.76, 20.20)	 0.047 0.004
Fibrosis • 0 (ref) • 1 • 2 • 3 • 4	<0.001~	 4.21 (0.38, 46.48) 5.47 (0.49, 60.50) 22.22 (2.55, 193.89) 193.06(25.23, 1477.1)	 3.11 (0.28, 34.73) 3.98 (0.36, 44.10) 17.54 (1.98, 155.01) 96.98 (12.44, 756.29)	 0.356 0.261 0.010 <0.001
Advanced Fibrosis	<0.001	26.51 (9.73, 72.20)	21.68 (7.76, 60.58)	<0.001
~Overall Comparison. *Adjusted for Age.				

A high ballooning score at baseline also remained significant in predicting liver mortality after age-control (aHR 5.96, 95% CI 1.76, 20.20, p=0.004). As fibrosis score was "not proportional" it could not be added directly to the model as a control, however as advanced fibrosis was proportional it could be used. After controlling for the presence of advanced fibrosis (F3-4) ballooning was no longer a significant independent predictor, and its presence was not found to be significant at controlling for advanced fibrosis stage which remained significant.

As with clinical outcomes, T2DM was added to the model with fibrosis to assess for any impact this may have on controlling for risk of liver mortality and was found to be not significant (p=0.621).

5.7 Summary of Chapter Findings

This chapter has explored the 42 patients who met the criteria of "liver mortality" during the follow-up period of the study, defined as a liver related death or transplantation. Several factors at baseline were found to independently predict liver mortality in this group, and as

may be expected, the greatest predictor of liver mortality at baseline was the presence of advanced fibrosis (p<0.001). However, there were several other variables identified which have been less reported in the general literature including GGT (p<0.001), the non-invasive scoring tools (p<0.001 for each tool) and a diagnosis of IHD (p=0.004).

5.8 Discussion

In this chapter factors associated with liver-specific mortality in the Newcastle NAFLD cohort were explored in greater detail than is available in much of the existing literature and therefore can provide important insight into the risk factors associated with adverse outcomes in this group of patients.

The factor associated with the greatest risk of liver specific mortality in the Newcastle cohort was the presence of advanced fibrosis (F3-4) at baseline. Fibrosis stage has consistently been shown to be the strongest predictor of all cause and liver specific mortality in patients with NAFLD.[97-101, 253, 254] There have been conflicting reports on other histological factors independently predicting outcomes. Initial studies suggested the presence of NASH or higher NAS scores may be associated with adverse outcomes. [36, 257] However, these findings are not consistently reported in the literature. In the Newcastle cohort, after controlling for fibrosis, there were no other histological markers significantly associated with liver mortality which is in keeping with a recent meta-analysis of 13 long-term outcome studies by Taylor and colleagues.[101] One of the major reasons for these differing findings is likely to be due to poor concordance between pathologists when grading histological features such as ballooning or inflammation. Brunt and colleagues[218] highlighted this discord in a recent study which identified there was what they described as “substantial divergence” in the reporting of hepatocyte ballooning, whilst Davison *et al*[219] demonstrated poor kappa scores between 3 pathologists reporting lobular inflammation scores (0.328). This lack of agreement in scoring histological markers will undoubtedly have an impact on the use of NASH or scores such as NAS or SAF in prognostication. Other factors such as sampling variability and relapsing-remitting nature of NASH features will also contribute to this.

The relationship between T2DM and NAFLD is known to be a complex and “bidirectional” one, with the presence of T2DM known to increase the risk of disease progression and the development of advanced fibrosis in patients with NAFLD.[23, 24] The Newcastle NAFLD cohort

has also demonstrated an associated increased risk in liver-related mortality in patients with T2DM, aHR 2.50 (95% CI 1.24 – 5.05, p=0.011). There are several other studies that have concluded that diabetes is an independent predictor of overall mortality in NAFLD[97, 349, 350], for example, a recent German population-based study with over 200,000 patients found T2DM was associated with a hazard ratio 1.32 (95% CI 1.29 – 1.34, p<0.001) for all-cause mortality.[351] However, few studies have looked at the relationship between diabetes and liver specific mortality. A smaller cohort study by Stepanova and colleagues[36], had findings in keeping with the Newcastle cohort, demonstrating that T2DM was an independent predictor of liver-related mortality with aHR 2.19 (95% CI 1.00 – 4.81). This independent risk for liver mortality is expected given the correlation between diabetes and more advanced disease, but it is an important risk that can be managed clinically by achieving good diabetic control, which therefore should remain a focus of clinical management.

It is well documented that cardiovascular disease is a leading cause of mortality in patients with NAFLD. Both cardiovascular disease and NAFLD share several common co-morbidities such as obesity, hypertension and the metabolic syndrome, but there is also emerging evidence to suggest that the relationship is more complicated than shared metabolic risk factors.[237, 350] The pro-inflammatory state and increased levels of cytokines and other such factors associated with steatosis and NASH are thought to contribute to the development of atherosclerosis and ischaemic heart disease, but the relationship remains incompletely understood.[12, 237, 352] The Newcastle NAFLD cohort demonstrates the presence of cardiovascular disease is a risk factor for liver-related mortality with an adjusted hazard ratio of 3.86 (95% CI 1.53 – 9.74, p=0.004). On reviewing the literature, cardiovascular disease is reported as an independent predictor of all-cause mortality in NAFLD in various studies[351] however, there appears to be a dearth of research exploring any association with liver-specific death in the NAFLD cohort. Given the increasing prevalence of NAFLD, associated increase in hospital referrals and resultant strain on healthcare to follow-up patients with NAFLD in the longer-term it is important to identify factors which may make a patient higher risk for an adverse liver outcome, especially in those without cirrhosis at the time of diagnosis. Further research is warranted into exploring this potential increased risk of liver related mortality in patients with NAFLD, as the presence of cardiovascular disease could form part of a risk stratification to identify those who should remain under secondary care follow-up.

As discussed in chapter 4, where all-cause mortality was explored, there have been various studies that have shown the potential use of the non-invasive scoring tools to predict long term outcomes such as overall mortality.[254, 353, 354] In this current chapter, a more detailed exploration of the use of these tools to predict liver specific mortality was undertaken. In the Newcastle NAFLD cohort using the low, indeterminate, and high categories to differentiate patients it can clearly be seen, when using the FIB-4 score or NFS, that those who fall into the low-risk category have almost a 100% survival free from liver mortality at 10 years. Whilst it is important to note that these results come from a highly selected cohort rather than a general population, these findings are highly significant in managing patients referred to secondary care with a diagnosis of NAFLD. The use of these simple, inexpensive, and readily available tools to risk stratify patients at an increased risk of a liver related death can confidently allow these patients to be followed-up in primary care with advice regarding life-style modifications. This approach is currently being used in the U.K. to identify patients at risk of advanced fibrosis and has been found to be cost-effective in managing the growing epidemic of NAFLD patients.[326, 355]

Patients who fall into the "high-risk" category are more likely to have advanced fibrosis or cirrhosis and therefore should remain under secondary care for regular screening for complications such as HCC, and therefore the question remains about the most appropriate management of those who fall into the "indeterminate" category. From the survival curves it can be seen that survival in these patients remains >90% at 10 years and therefore it has been proposed that the use of repeat non-invasive scores could help monitor for disease progression and the associated risk of liver related mortality.

On reviewing the literature there are very few studies that explore the use of non-invasive tools to identify those specifically at risk of liver related mortality. In keeping with the Newcastle cohort, Kim *et al*[356] also found that patients with "high risk" NFS scores had a small increased risk of liver specific death (aHR 1.07, 95% CI 0.00 – 1.25, p=0.070) but did not find the FIB-4 results to be significant at predicting increased risk (p=0.821). One of the major limitations of this study however was the very small number of liver deaths (n=19) and therefore it is difficult to draw meaningful conclusions from their results. Unalp-Arida and colleagues[357] undertook a population-based study, excluding only those with known viral hepatitis, which found that patients with indeterminate or high FIB-4 or NFS scores were associated with increased liver disease mortality. More recently Boursier *et al*[326]

demonstrated the FIB-4 score was able to identify patients with NAFLD at a greater risk of a liver related event, which included HCC, decompensation, and liver mortality. Systematic reviews by Liu *et al*[358] and Lee *et al*[359] also agree with the findings in these studies. The Newcastle NAFLD cohort can add further evidence to the use of non-invasive scores, in particular FIB-4 and NFS, to stratify patients with NAFLD at risk of a liver related death. These findings can assist in the practical management of an increasingly prevalent disease however, further study in their clinical use is required as some studies are proposing the use of alternative cut-offs for the purposes of identifying mortality risk rather than the presence of advanced fibrosis as the tools were originally developed for. Population based studies are also required to establish the role of the scores in a primary care setting.

In summary, this chapter has examined those within the Newcastle NAFLD cohort who died of a liver related death or received a liver transplant. These findings do have several limitations, for example, the small number of individuals who reached a liver mortality outcome, and these limitations will be fully examined in chapter 8. Fibrosis stage was found to be the greatest independent predictor of mortality within this cohort, which is in keeping with the general literature. Other features, such as T2DM and IHD, were also shown to be associated with an increased risk of liver mortality. The patients who fell into the “high risk” categories of the FIB-4 and NFS scores were also found to be at an increased risk of death which has not yet been widely explored in the literature and these findings will add to this body of evidence. Other liver outcomes such as progression to cirrhosis and the development of HCC will be explored in the next chapter.

Chapter 6: Liver related Outcomes

6.1. Introduction

Non-alcoholic fatty liver disease has become a leading cause of chronic liver disease, particularly in the Western world, with the global prevalence estimated to be 24%.[50] Rising rates of obesity, including in children, and aging populations are expected to see this prevalence increase in years to come with some models predicting a 20.2% increase in the U.K. by 2030.[323] Despite the slow progression to advanced fibrosis and cirrhosis, affecting 15-30% of NAFLD patients,[96] the increasing prevalence of the disease will inevitably lead to an increase in complications of cirrhosis. One study has estimated an increase in decompensated cirrhosis cases by 180% and a 137% increase in the incidence of HCC in the United States over a 15-year period from 2015-2030.[82] This rise in hospital referrals for assessment and monitoring NAFLD as well as managing patients with end stage liver disease and complications will create a significant increase in the healthcare and economic burden of NAFLD. One study used Markov modelling to predict an increase in the associated annual cost across four European countries including Germany, Italy, France, and the U.K. from €35 billion (£5.24 billion in the UK) to €334 billion annually over a 10-year period.[360] In order to prevent disease progression and prepare for the increased burden on healthcare further work needs to be undertaken to understand the natural history of NAFLD and factors which may predict events such as the progression of fibrosis and development of cirrhosis, HCC and the need for liver transplantation.

6.2 Chapter Aims

This chapter will:

1. Explore the group of patients within the cohort who were found to have disease progression on sequential biopsies and review any factors that may be prognostic for histological progression.
2. Explore the patients in the cohort that clinically progressed to cirrhosis during the study and examine any factors that may be prognostic for clinical progression to cirrhosis.

3. Review the frequency and clinical characteristics of patients who were diagnosed with a Hepatocellular carcinoma during follow-up and any factors that may prognosticate this.
4. Describe the characteristics of those who received a liver transplant during the study.

6.3 Disease progression- Histological

In 2015 McPherson and colleagues published a study reviewing paired histology on a cohort of 108 patients.[33] This provided important evidence challenging the previously held idea that patients with NAFL had a benign and non-progressive disease course and identified the presence of T2DM as a predictor for disease progression. This study used patients from the Newcastle NAFLD service and since its publication there have been a further 33 patients who have undergone a repeat liver biopsy, and an additional 5 years of follow-up.

This section will therefore focus on the original "Delta" cohort in addition to the newer patients and will explore any features which may be prognostic of progression.

6.3.1. Definition

During the follow-up period there were 141 patients who underwent more than one liver biopsy, accounting for 23.5% of the entire Newcastle NAFLD cohort. There are many reasons for undertaking a repeat biopsy and many different histological markers that can be monitored for disease change including the presence or absence of NASH, NAS score, SAF activity score and fibrosis stage. As fibrosis stage has consistently been shown to be the only significant predictor of outcomes in NAFLD[97, 98, 253], a change in fibrosis stage between liver biopsies will be the main focus of this paired biopsy review.

Of the 141 patients, 16 (11.3%) were shown to have improvement in their fibrosis stage, 61 (43.3%) remained at the same fibrosis stage and 64 (45.4%) had a fibrosis score which had progressed from their original biopsy results as demonstrated in figures 6.1 and 6.2 below. Table 6.1 will also demonstrate the movement of fibrosis stage by each stage at baseline. This section will explore those who had a progression in histological fibrosis over time.

Figure 6.1. Change in histology over time in repeat biopsies.

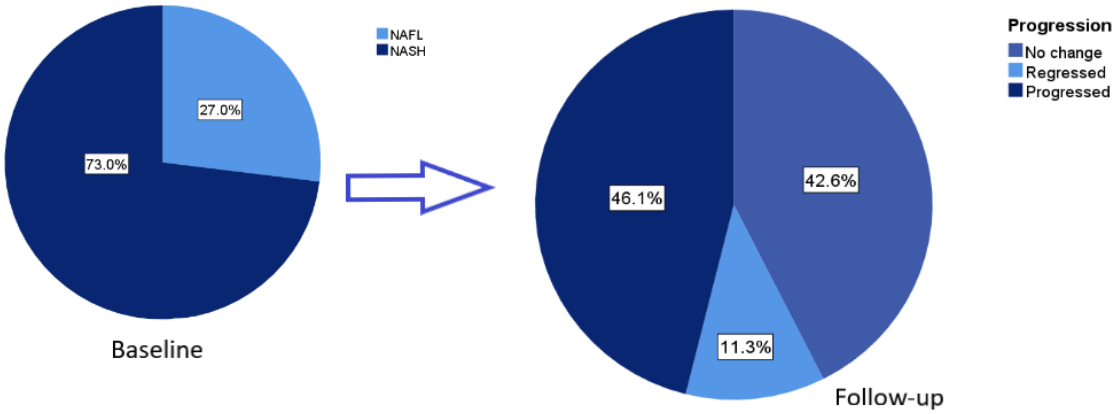


Figure 6.2. Sankey diagram demonstrating fibrosis change by ≥1 stage over time. From this graph it can be seen that some patients had an increase in fibrosis by up to 3 stages over time (F0-3).

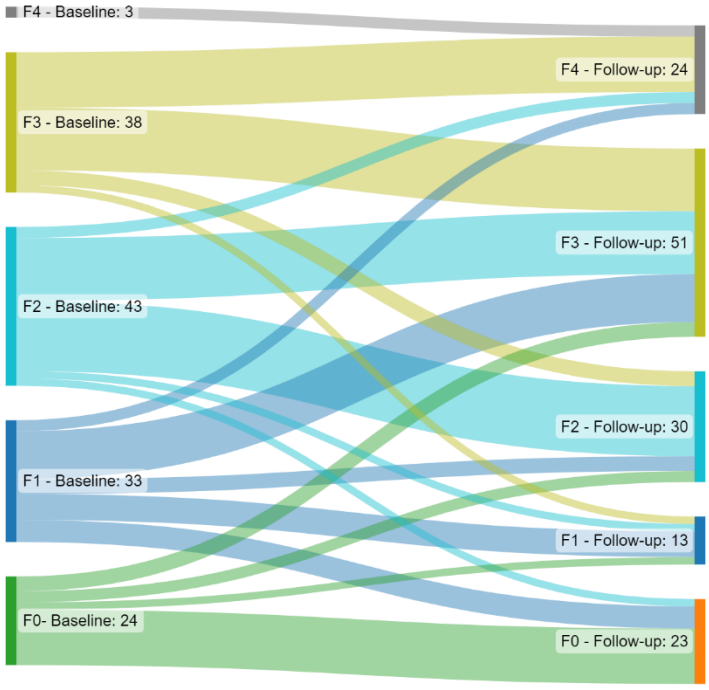


Table 6.1. Movement of fibrosis change between biopsies stratified by baseline fibrosis stage.

Baseline	Follow-up				
	F0	**F1**	**F2**	**F3**	**F4**
F0	15	2	3	4	0
F1	6	7	4	13	3
F2	2	2	19	17	3
F3	0	2	4	17	15
F4	0	0	0	0	3

6.3.2 Characteristics of histological progressors

Table 6.2 below details the baseline and follow-up characteristics of interest in the group of patients who had histological progression on paired biopsies over the course of the study.

The mean age at the first liver biopsy was 50 ± 12 years old and 59.4% (38) of the group were men. At baseline there were elevated levels of ALT, AST and GGT in this group and 54.0% (34) of the group had a diagnosis of T2DM at this stage.

The median time between biopsies was 6.9 years (1.3-23.1). Over the course of the study there was a significant reduction in mean ALT ($p<0.001$), AST ($p=0.008$) and Platelet levels ($p<0.001$). There was a notable increase in IgA levels ($p=0.028$) and the results of the non-invasive tools also increased over time in keeping with more advanced liver disease. The prevalence of co-morbidities also increased over the follow-up period and by the time of the repeat biopsy 81.0% had a diagnosis of T2DM.

There was no significant change in mean BMI, Albumin or PT results over the follow-up period and no significant increase in the occurrence of heart disease.

Table 6.2. Baseline and follow-up characteristics of histological progressors. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Baseline n=64	Follow-up n=64	p value
Age (years)	50 ± 12	59 ± 12	<0.001#
BMI (kg/m^2)	34.0 (24.0-50.0)	35.7 ± 5.3	0.346*
ALT (0 – 40) [U/l)	76 (29-308)	71 ± 51	<0.001*
AST (0 – 40) [U/l)	55 (21-225)	30 (18-96)	0.008*
GGT (0 – 70) [U/l)	84 (19-691)	58 (10-200)	0.079*
Albumin (35 – 50) [g/l]	46 (39-55)	45 (31-51)	0.157*
Platelets (150 – 450) [x10^9/l]	238 ± 65	213 ± 71	<0.001#
PT (10 – 13) [seconds]	12 (9-14)	12 (10-32)	0.633*
IgA (0.8 – 3.0) [g/l]	2.63 (1.00-6.79)	3.33 ± 1.58	0.028*
FIB-4	1.26 (0.51-6.36)	1.45 (0.41-8.37)	0.009*
NFS	-0.98 ± 1.29	-0.15 ± 1.35	<0.001#
T2DM	34 (54.0%)	51 (81.0%)	<0.001~
HTN	27 (50.0%)	39 (69.6%)	0.039~
MetS	25 (56.8%)	42 (84.0%)	<0.001~
IHD	4 (18.2%)	12 (21.8%)	1.00~
Cirrhosis	0	21 (37.5%)	<0.001~
*Wilcoxon signed rank test, #Paired t test, ~McNemar's change test			

The histological features of those who had disease progression between the paired biopsies are described in table 6.3 below. There were significant changes across each of the histological components with time but of note there was a large increase in the number of patients with advanced fibrosis (21.9% vs 85.9%, p<0.001). The histological diagnosis of "NAFL" was present in 23.4% of the patients" at baseline.

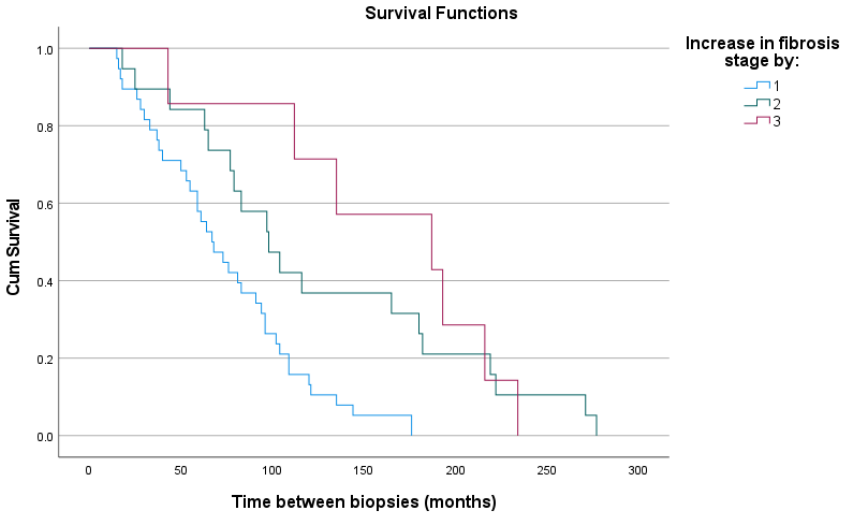
Table 6.3. Comparison of baseline and repeat liver biopsy results for those with histological progression. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	Baseline n=64	Follow-up n=64	p value
Steatosis • 0 • 1 • 2 • 3	 0 3 (4.7%) 38 (59.4%) 23 (35.9%)	 0 10 (15.6%) 42 (65.6%) 12 (18.8%)	0.001*
Ballooning • 0 • 1 • 2	 14 (21.9%) 32 (50.0%) 18 (28.1%)	 4 (6.3%) 28 (43.8%) 32 (50.0%)	0.001*
Inflammation • 0 • 1 • 2 • 3	 12 (18.8%) 36 (56.3%) 14 (21.9%) 2 (3.1%)	 3 (4.7%) 32 (50.0%) 25 (39.1%) 4(6.3%)	0.004*
Fibrosis • F0 • F1 • F2 • F3 • F4	 10 (15.6%) 20 (31.3%) 20 (31.3%) 14 (21.9%) 0	 0 2 (3.1%) 7 (10.9%) 34 (53.1%) 21 (32.8%)	<0.001*
Fibrosis category • No significant (0-1) • Clinically significant (2-4) • Advanced (3-4)	 30 (46.9%) 34 (53.1%) 14 (21.9%)	 2 (3.1%) 62 (96.9%) 55 (85.9%)	 <0.001* <0.001*
NASH (present)	48 (75.0%)	57 (89.1%)	0.059*
NAS Score Categories • 1-2 • 3-4 • ≥5	 7 (10.9%) 26 (40.6%) 31 (48.4%)	 3 (4.7%) 24 (37.5%) 37 (57.8%)	0.133*
SAF Activity Score	2 (0-4)	3 (0-4)	0.003*
Fibrosing steatohepatitis	33 (51.6%)	57 (89.1%)	0.039*
*Wilcoxon signed rank test, ~Fisher's exact test			

Of the 64 patients with fibrosis progression, 7 (10.9%) had an increase of 3 fibrosis stages between biopsies. The mean time between biopsies in these patients was 160 ± 67 months,

which was substantially longer than those who only progressed 1 or 2 stages (74 and 126 months respectively) as can be seen in figure 6.3 below.

Figure 6.3. Kaplan Meier curves demonstrating the time between biopsies stratified by increase in fibrosis stage. From the curves it can be seen that those who progressed by only 1 stage had the shortest time in between biopsies.



At the time of the baseline biopsy only 1 of these patients (14.3%) met the criteria for NASH, but this increased to 100% by the time of the repeat histology (p=0.031).

Over the course of the study none of the patients that had histological progression to cirrhosis received a liver transplant and 9 patients died. Three (33.3%) were liver related deaths: 2 patients died of decompensated NASH cirrhosis and 1 from hepatocellular carcinoma. Of the remaining patients 2 died from cardiovascular disease, 2 from malignancy and 2 of "other" causes.

6.3.3. Comparison to those who did not progress histologically.

In order to identify any factors that may be associated with disease progression, a comparison was made between those who had histological progression on repeat biopsy and those who did not. For this analysis the group of "non-progressors" will include both those who had no change in their fibrosis stage between biopsies (n=61) and those who had improvement in fibrosis (n=16).

Table 6.4 below displays the baseline characteristics of those who progressed histologically compared to those who did not. Those who progressed were older at the time of the initial biopsy (50 vs 48yrs), but this was not found to be significant and there was no difference in

BMI (p=0.461) or sex (p=0.601) between the groups. There were no significant differences in baseline blood results between the groups, but those who progressed had significantly higher non-invasive tool results (FIB-4 p=0.034, NFS p=0.011). There was also no significant difference in the prevalence of co-morbidities at the time of the index liver biopsy.

Table 6.4. Comparison of baseline characteristics between those who had histological progressor groups. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Progressor n=64	Non-progressor n=77	p value
Duration of follow-up (months)	83 (15-247)	81 (8-198)	0.162^{+}
Age (years)	50 ± 12	48 (18-64)	0.056^{+}
Sex (male)	38 (59.4%)	50 (64.9%)	0.601*
BMI (kg/m^2)	34. 0 (24.0-50.0)	33.0 (24.0-59.1)	0.461^{+}
ALT (0 – 40) [U/l)	76 (29-308)	76 (15-355)	0.912^{+}
AST (0 – 40) [U/l)	55 (21-225)	53 (19-229)	0.237^{+}
GGT (0 – 70) [U/l)	84 (19-691)	71 (17-670)	0.406^{+}
Albumin (35 – 50) [g/l]	46 (39-55)	45 ± 3	0.926^{+}
Platelets (150 – 450) [x10^9/l]	238 ± 65	255 ± 66	0.142~
PT (10 – 13) [seconds]	12 (9-14)	12 (10-15)	0.559^{+}
IgA (0.8 – 3.0) [g/l]	2.63 (1.00-6.79)	2.61 (0.86-7.68)	0.743^{+}
FIB-4 Score	1.26 (0.51-6.36)	1.07 (0.31-3.04)	0.034^{+}
NFS	-0.98 ± 1.29	-1.89 (-5.14-4.27)	0.011^{+}
T2 DM	34 (54.0%)	29 (38.7%)	0.087*
HTN	27 (50.0%)	34 (50.0%)	1.00*
MetS	25 (56.8%)	23 (43.4%)	0.224*
IHD	4 (18.2%)	2 (4.5%)	0.090$^{\#}$
$^{+}$Mann Whitney U, *Chi Square, #Fisher's Exact, ~Student t test			

A comparison between the baseline histology results of both groups can be seen in table 6.5 below. The only biopsy feature which was significantly different between the two groups at this stage is those who progressed were found to have a higher steatosis score (p=0.002).

Table 6.5. Comparison of index biopsy results between histological progressor groups. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	Progressors n=64	Regress/No progress n=77	p value
Steatosis			0.002*
• 0	0	0	
• 1	3 (4.7%)	23 (29.9%)	
• 2	38 (59.4%)	35 (45.5%)	
• 3	23 (35.9%)	19 (24.7%)	
Ballooning			0.110*
• 0	14 (21.9%)	20 (26.0%)	
• 1	32 (50.0%)	44 (57.1%)	
• 2	18 (28.1%)	13 (16.9%)	
Inflammation			0.937*
• 0	12 (18.8%)	18 (23.4%)	
• 1	36 (56.3%)	33 (42.9%)	
• 2	14 (21.9%)	25 (32.5%)	
• 3	2 (3.1%)	1 (1.3%)	
Fibrosis			0.306*
• F0	10 (15.6%)	15 (19.5%)	
• F1	20 (31.3%)	13 (16.9%)	
• F2	20 (31.3%)	23 (29.9%)	
• F3	14 (21.9%)	23 (29.9%)	
• F4	0	3 (3.9%)	
Fibrosis category			
• No significant (0-1)	30 (46.9%)	28 (36.4%)	0.305#
• Clinically significant (2-4)	34 (53.1%)	49 (63.6%)	
• Advanced (3-4)	14 (21.9%)	26 (33.8%)	0.197#
NASH (present)	48 (75.0%)	54 (70.1%)	0.448#
NAS Score Categories			0.176*
• 1-2	7 (10.9%)	16 (20.8%)	
• 3-4	26 (40.6%)	28 (36.4%)	
• ≥5	31 (48.4%)	33 (42.9%)	
SAF Activity Score	2 (0-4)	2 (0-4)	0.480*
Fibrosing steatohepatitis	33 (51.6%)	53 (68.8%)	0.457#
*Kruskal-Wallis test, #Chi square test			

6.3.4. Factors predicting progression.

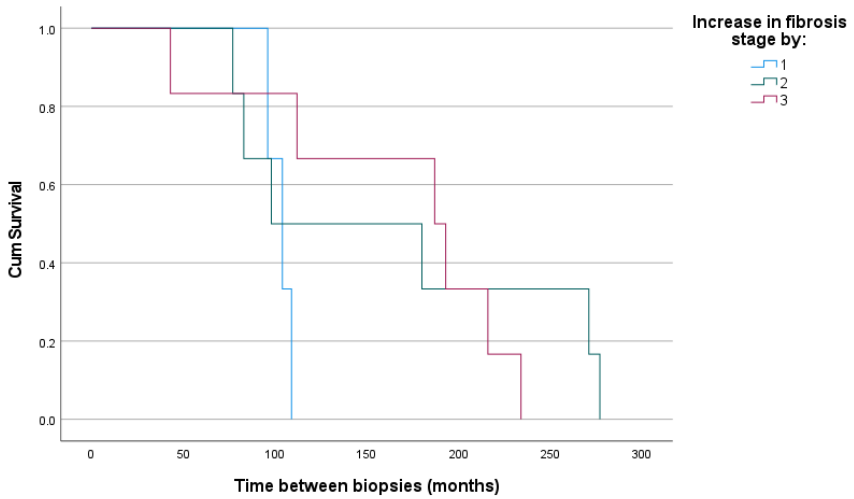
The above factors that were significant on univariate analysis were then incorporated into multivariate binary regression. This included Age, FIB-4 score, NFS, T2DM, IHD and Steatosis.

The presence of T2DM at baseline was shown to be an independent predictor for disease progression (OR: 4.2, 95% CI 1.16 – 14.92, p=0.029) and a Steatosis score of 3 compared to 1 was also found to be a predictor for histological progression (OR 14.04, 95% CI 1.18 – 167.33, p=0.037).

6.3.5 NAFL progressors

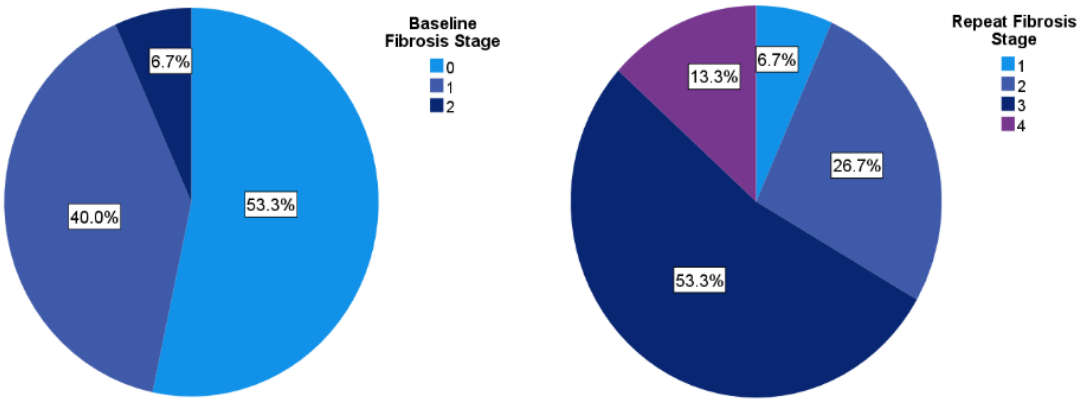
Fifteen of the sixty four with fibrosis progression only had "NAFL" at baseline. Three progressed 1 stage with a mean time of 103 months between biopsies, 6 progressed 2 stages with a mean time of 164 months between biopsies and 6 progressed 3 stages with a mean time of 164 months between biopsies, as shown in figure 6.4 below. Two thirds (10) of the NAFL progressor group were men and the mean age at the time of the index biopsy was 45 ± 14 years old.

Figure 6.4. Kaplan Meier curves demonstrating the time between biopsies stratified by the number of fibrosis stage increase in time in the NAFL group. From the curves it can be seen that those who progressed by only 1 stage had the shortest time in between biopsies.



By the time of the repeat histology 2 patients (13.3%) had progressed to F4 and 13 (86.7%) met the criteria for NASH. Figure 6.5 below demonstrates the distribution of fibrosis stage at baseline and follow-up liver biopsy.

Figure 6.5. Fibrosis stages at baseline and follow-up in NAFL group.



When comparing to the group of NASH progressors the time between biopsies was significantly longer in the NAFL group (152 months vs 83 months) which may account for the higher rate of progression in the NAFL group ($p<0.001$). There was no significant difference in any of the clinical baseline characteristics between the groups. Histologically there were significant differences in ballooning, inflammation, fibrosis stages, NAS categories and SAF activity scores between the groups ($p<0.001$ for all) but this is to be expected given the definition of NASH.

6.4 Disease progression- Clinical

6.4.1 Definition of group

Over the course of the study there were 50 patients newly diagnosed with cirrhosis during the follow-up period. Fifteen (30%) of these new cirrhosis cases were diagnosed based on repeat liver histology, 23 (46%) had radiology imaging in keeping with cirrhosis (CT or AUSS), 6 (12%) had a Fibroscan™ which led to the diagnosis of cirrhosis and the remaining 6 (12%) were clinically diagnosed after an episode of decompensated cirrhosis.

This section will review all of the patients that had liver disease progression during the follow-up period, including those diagnosed by means other than liver biopsy.

6.4.2 Characteristics of clinical progressors to cirrhosis

The baseline and follow-up characteristics of the group of patients that clinically progressed to cirrhosis during the study were explored and are detailed in table 6.6 below.

Over half (54.0%, 27) of the group were male and the median age at the time of the baseline visit was 56 years (19-72). Elevated ALT, AST and GGT levels were seen at the first clinical visit and 38.8% had T2DM.

The median follow-up for this group of patients was 13.3 years (4.8-35.4) and over the course of the follow-up there was a significant reduction in ALT ($p<0.001$), AST ($p<0.001$), Albumin ($p<0.001$) and Platelet levels ($p<0.001$) in keeping with more advanced liver disease at the end of the study. There was also a significant increase in the FIB-4 results ($p=0.004$) which would be expected with progressive fibrosis, but there was no significant change in NFS results over time. The proportion of patients with co-morbidities increased overall and in particular T2DM was present in 85.4% (from 38.8%, $p<0.001$).

Table 6.6. Baseline and follow-up characteristics of those who clinically progressed to cirrhosis during the study. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Baseline n=50	Follow-up n=50	p value
Age (years)	56 (19-72)	70 (30-87)	<0.001*
BMI (kg/m^2)	34.7 (26.5-50.0)	32.9 ± 5.0	0.181*
ALT (0 – 40) [U/l)	74 (24-182)	33 (11-85)	<0.001*
AST (0 – 40) [U/l)	55 (28-225)	37 (17-96)	<0.001*
GGT (0 – 70) [U/l)	88 (26-717)	86 (29-532)	0.831*
Albumin (35 – 50) [g/l]	45 (39-55)	44 (27-51)	0.001*
Platelets (150 – 450) [x10^9/l]	228 ± 68	183 ± 82	<0.001#
PT (10 – 13) [seconds]	12 (9-14)	13 (10-17)	0.002*
IgA (0.8 – 3.0) [g/l]	2.84 (1.18-6.38)	2.79 (1.46-7.04)	0.225*
AST/ALT Ratio	0.78 (0.51-1.63)	0.95 (0.57-2.14)	<0.001*
APRI	0.68 (0.26-2.80)	0.51 (0.16-1.83)	0.501*
FIB-4	1.68 (0.50-6.36)	2.08 (0.51-8.37)	0.004*
NFS	-0.72 ± 1.40	-0.32 (-1.21-2.85)	0.273*
T2DM	19 (38.8%)	41 (85.4%)	<0.001~
HTN	25 (59.5%)	36 (76.6%)	0.109~
MetS	27 (75.0%)	29 (90.6%)	0.063~
IHD	6 (24.0%)	16 (43.2%)	0.125~
*Wilcoxon signed rank test, #Paired t test, ~McNemar's change test			

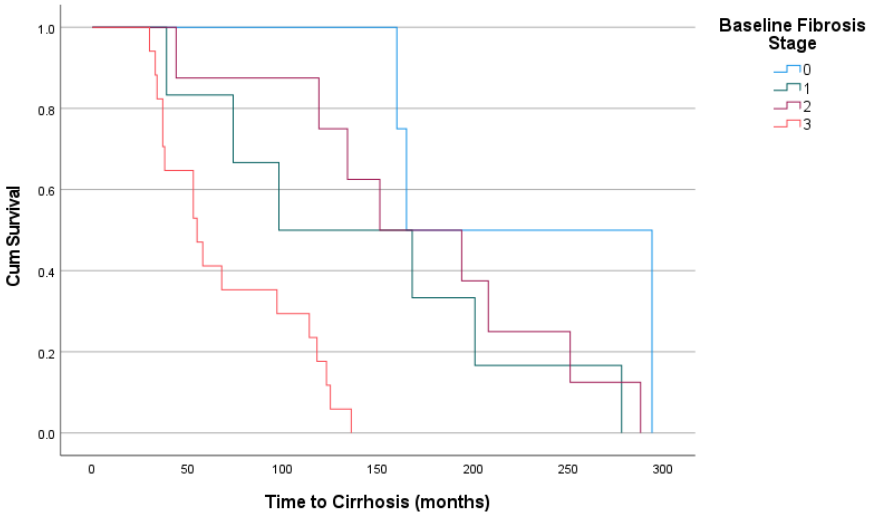
Table 6.7 below documents the baseline histological findings of the group who progressed to cirrhosis by the end of the study. The majority of these patients had NASH (80.0%) with a high NAS score (58.0% ≥5) and 68.0% met the criteria for fibrosing steatohepatitis. Of note, 70.0% of this group were found to have clinically significant fibrosis (F2-4) at the time of the initial liver biopsy.

Table 6.7. Baseline histology of clinical progressors to cirrhosis. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	Baseline
Steatosis • 0 • 1 • 2 • 3	 0 3 (6.0%) 28 (56.0%) 19 (38.0%)
Ballooning • 0 • 1 • 2	 7 (14.0%) 30 (60.0%) 13 (26.0%)
Inflammation • 0 • 1 • 2 • 3	 8 (16.0%) 22 (44.0%) 17 (34.0%) 3 (6.0%)
Fibrosis • F0 • F1 • F2 • F3 • F4	 5 (10.0%) 10 (20.0%) 12 (24.0%) 23 (46.0%) 0
Fibrosis category • No significant (0-1) • Clinically significant (2-4) • Advanced (3-4)	 15 (30.0%) 35 (70.0%) 23 (46.0%)
NASH (present)	40 (80.0%)
NAS Score Categories • 1-2 • 3-4 • ≥5	 6 (12.0%) 15 (30.0%) 29 (58.0%)
SAF Activity Score	2 (0-4)
Fibrosing steatohepatitis	34 (68.0%)

The mean time to a diagnosis of cirrhosis from the baseline visit was 10.0 ± 7.0 years (112 ± 84 months). Unsurprisingly those with F3 on the baseline biopsy were noted to progress to cirrhosis quickest with a mean time to cirrhosis in 70 ± 38 months compared to 228 ± 76 months in patients with F0, as can be seen in figure 6.6 below.

Figure 6.6. Kaplan Meier curve demonstrating time to cirrhosis diagnosis stratified by baseline fibrosis stage. From the curves it can be seen those with F3 at baseline had the shortest time to a diagnosis of cirrhosis.



Of the 50 patients newly diagnosed with cirrhosis during the study, none received a liver transplant and 12 (24.0%) died by the end of the follow-up. The most common cause of death was liver related accounting for 7 (58.3%) of the deaths: 4 due to decompensated NASH cirrhosis and 3 from hepatocellular carcinoma. The remaining patients died of cardiovascular disease (2), malignancy (1) and "other" cause of death including frailty (2).

6.4.3. Comparison to those who did not progress to cirrhosis.

To identify any factors associated with clinical progression to cirrhosis those who were found to progress during follow-up were compared to those who did not clinically progress to cirrhosis by the end of the study. Table 6.8 below describes the baseline characteristics of these groups. It is worth noting there is a difference in the size of these groups, and this should be taken into consideration when reviewing the comparative results. The "non-progressors" group excludes those who were diagnosed with cirrhosis at baseline and patients who were lost to follow-up and therefore could not have progression confirmed or refuted.

The "progressors" were older at baseline (56 vs 51 years old) and there was no significant difference in the duration of follow-up (p=0.576). The distribution of sex between the groups was similar (54.0% vs 63.1% male) and there was no significant difference in BMI (p=0.149).

Baseline liver bloods showed those who progressed had higher AST (<0.001) and GGT levels (0.028), with lower platelet counts (p=0.006). Of note, the non-invasive tool results were all higher in the group that progressed at baseline, and all of these results are in keeping with

more advanced disease in this group overall. There was no difference in the number of patients with diabetes (38.8% vs 37.8%) but those who progressed trended towards having an increased incidence of HTN (59.5% vs 44.2%, p=0.070).

Table 6.8. Comparison of baseline characteristics between those who clinically progressed to cirrhosis vs those who did not. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Clinical progressor n= 50	No progression n= 339	p value
Duration of follow-up (months)	159 (57-425)	169 (12-359)	0.576⁺
Age (years)	56 (19-72)	51 (18-76)	0.010⁺
Sex (male)	27 (54.0%)	214 (63.1%)	0.275*
BMI (kg/m^2)	34.7 (26.5-50.0)	33.5 (23.3-59.1)	0.149⁺
ALT (0 – 40) [U/l)	74 (24-182)	62 (13-355)	0.139⁺
AST (0 – 40) [U/l)	55 (28-225)	41 (13-251)	<0.001⁺
GGT (0 – 70) [U/l)	88 (26-717)	73 (14-1141)	0.028⁺
Albumin (35 – 50) [g/l]	45 (39-55)	45 ± 3	0.421⁺
Platelets (150 – 450) [x10^9/l]	228 ± 68	258 ± 67	0.006~
PT (10 – 13) [seconds]	12 (9-14)	12 (9-15)	0.109⁺
IgA (0.8 – 3.0) [g/l]	2.84 (1.18-6.38)	2.51 (0.26-20.90)	0.229⁺
AST/ALT Ratio	0.78 (0.51-1.63)	0.69 (0.22-2.91)	0.008⁺
APRI	0.68 (0.26-2.80)	0.41 (0.08-3.18)	<0.001⁺
FIB-4	1.68 (0.50-6.36)	1.05 (0.15-7.43)	<0.001⁺
NFS	-0.72 ± 1.40	-1.79 ± 1.51	<0.001~
T2DM	19 (38.8%)	128 (37.8%)	1.00*
HTN	25 (59.5%)	137 (44.2%)	0.070*
MetS	27 (75.0%)	177 (70.5%)	0.696*
IHD	6 (24.0%)	32 (11.5%)	0.105*
⁺Mann Whitney U, *Chi Square, #Fisher's Exact, ~Student t test			

Histological features at baseline were also compared between the groups and shown in table 6.9 below. The group of "progressors" had higher scores for each of the individual

histological components suggesting more disease activity which was reflected in higher NAS (p<0.001) and SAF scores (p<0.001). Of particular note 46.0% of this group had advanced fibrosis compared to 17.7% of those who did not progress (p<0.001).

Table 6.9. Comparison of baseline histology between those who clinically progressed to cirrhosis vs those who did not. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	Clinically progressed n= 50	No progression n= 339	p value
Steatosis • 0 • 1 • 2 • 3	 0 3 (6.0%) 28 (56.0%) 19 (38.0%)	 0 110 (32.4%) 148 (43.7%) 81 (23.9%)	<0.001*
Ballooning • 0 • 1 • 2	 7 (14.0%) 30 (60.0%) 13 (26.0%)	 149 (44.0%) 139 (41.0%) 51 (15.0%)	<0.001*
Inflammation • 0 • 1 • 2 • 3	 8 (16.0%) 22 (44.0%) 17 (34.0%) 3 (6.0%)	 102 (30.1%) 160 (47.2%) 73 (21.5%) 4 (1.2%)	0.003
Fibrosis • F0 • F1 • F2 • F3 • F4	 5 (10.0%) 10 (20.0%) 12 (24.0%) 23 (46.0%) 0	 121 (35.7%) 73 (21.5%) 85 (25.1%) 60 (17.7%) 0	<0.001*
Fibrosis category • No significant (0-1) • Clinically significant (2-4) • Advanced (3-4)	 15 (30.0%) 35 (70.0%) 23 (46.0%)	 194 (57.2%) 145 (42.8%) 60 (17.7%)	 <0.001# <0.001#
NASH (present)	40 (80.0%)	179 (52.8%)	<0.001#
NAS Score Categories • 1-2 • 3-4 • ≥5	 6 (12.0%) 15 (30.0%) 29 (58.0%)	 108 (31.9%) 120 (35.4%) 111 (32.7%)	<0.001*
SAF Activity Score	2 (0-4)	2 (0-4)	<0.001*
Fibrosing steatohepatitis	34 (68.0%)	123 (36.3%)	<0.001#
*Kruskal-Wallis test, #Chi square test			

6.4.4 Factors predicting clinical progression to cirrhosis.

As with the predictive factors for histological progression above, multivariate binary regression was performed using the factors significant in univariate analysis comparing the clinical progressors. Given there were several significant factors this was initially split into 2 backwards models: one based on clinical variables (i.e., Age, AST, GGT, Plt, AST/ALT Ratio, APRI score, FIB-4 score, NFS and HTN) and the other on histological features.

Of the clinical factors the FIB-4 score was the only significant baseline characteristic which would predict clinical progression (OR: 7.04, 95% CI 1.64 – 30.17, p=0.009).

From the histological features there were three factors which were significant: Steatosis [score 3 vs 1 (OR 14.57, 95% CI 2.75 – 77.23, p=0.002)], Fibrosis [F3 vs F0 (OR 8.38, 95% CI 1.75 – 40.18, p=0.008)] and NAS categories [≥5 vs 1-2 (OR 0.87, 95% CI 0.01 – 0.77, p=0.028)].

Finally, these 4 factors were combined into another multivariate binary regression model and NAS categories were no longer significant. The results of this final multivariate analysis can be seen in table 6.10 below.

Table 6.10 Odds Ratios for predictors of clinical progression to cirrhosis by binary regression. Multivariate binary regression was undertaken for variables significantly associated with clinical progression in univariate analysis. This was split into 2 models: clinical variables (blood results, NITs) and histological variables. FIB-4 score, high steatosis grade, fibrosis stage and high NAS category (≥5) were all significant in the initial models and were thus combined in a further multivariate regression analysis. Steatosis, Fibrosis and FIB-4 remained significant in this model and ORs are displayed in the table below.

Variable	OR	95% CI	p value
Steatosis			
• 1 (ref)			
• 2	11.52	2.08 – 63.81	0.005
• 3	16.16	2.40 – 108.69	0.004
Fibrosis			
• 0 (ref)			
• 1	2.24	0.55 – 9.22	0.263
• 2	1.55	0.36 – 6.64	0.557
• 3	5.76	1.54 – 21.50	0.009
FIB-4	1.83	1.28 – 2.63	0.001

6.5 Hepatocellular Carcinoma

6.5.1 New diagnosis HCC

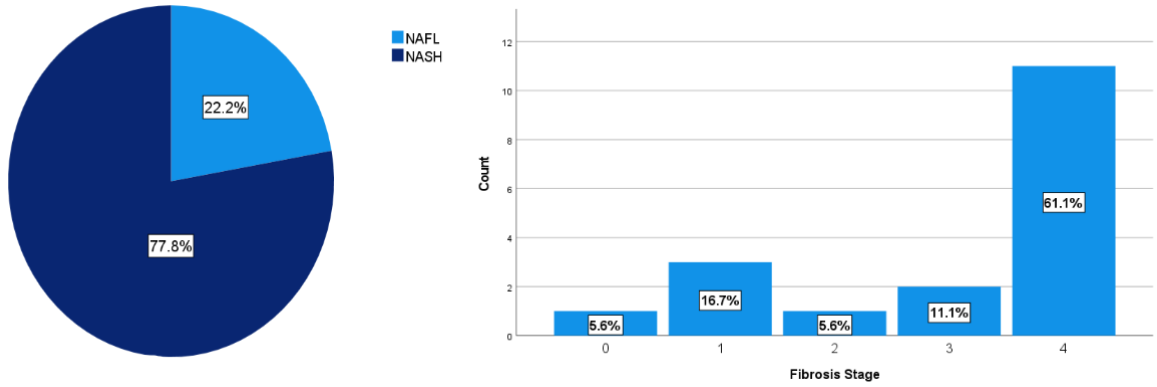
Over the course of the study there were 24 patients diagnosed with a new occurrence of hepatocellular carcinoma, accounting for 4.0% of the entire Newcastle NAFLD cohort. The majority of patients were male (58.3%) and the median age at the baseline visit was 60 years (26-80). The median results of the liver blood tests were elevated at the time of the baseline review (ALT, AST and GGT) and notably IgA levels were high (4.12 ± 2.25g/l). 52.2% (12) of this group had a diagnosis of T2DM at baseline and the incidence of IHD was also high compared to the entire cohort (46.2%).

The mean length of follow-up was 10.8 years (1.5-35.4) and over this time the mean liver blood test results notably decreased. As has been noted in previous chapters, there were incomplete data available for this group due to a number of reasons including a large proportion of the group had died by the final event (20) and blood results were not recorded in these cases. Full comparison of blood results and non-invasive scores is therefore not possible, but a trend of reduced mean liver blood results (ALT, AST and GGT) and an increase in mean NFS can be seen.

The prevalence of co-morbidities in the HCC group had notably increased with time, in particular T2DM 90% (from 52.2%), HTN 94.4% (from 37.5%), and IHD 58.8% (from 46.2%).

The baseline histology of the HCC group was also explored and there was a high prevalence of NASH (77.8%), 44.4% had a NAS score ≥5 and 72.2% had F3-4. Figure 6.7 below shows the distribution of those with NASH and fibrosis stage from baseline histology.

Figure 6.7. Baseline histology for those who developed HCC.



Three of the patients in the HCC group (12.5%) received a liver transplant during the follow-up period, with HCC as the clinical indication for transplant. The mean time to transplant was 5.2 ± 2.0 years (62 ± 24 months).

The vast majority of patients with a diagnosis of HCC died during the course of the study (20, 83.3%). 12 (60%) died as a result of HCC and 4 (20%) from decompensated NASH cirrhosis. The remaining 4 died of "other" causes including frailty and infection. 1 patient who received a transplant died as a result of infection.

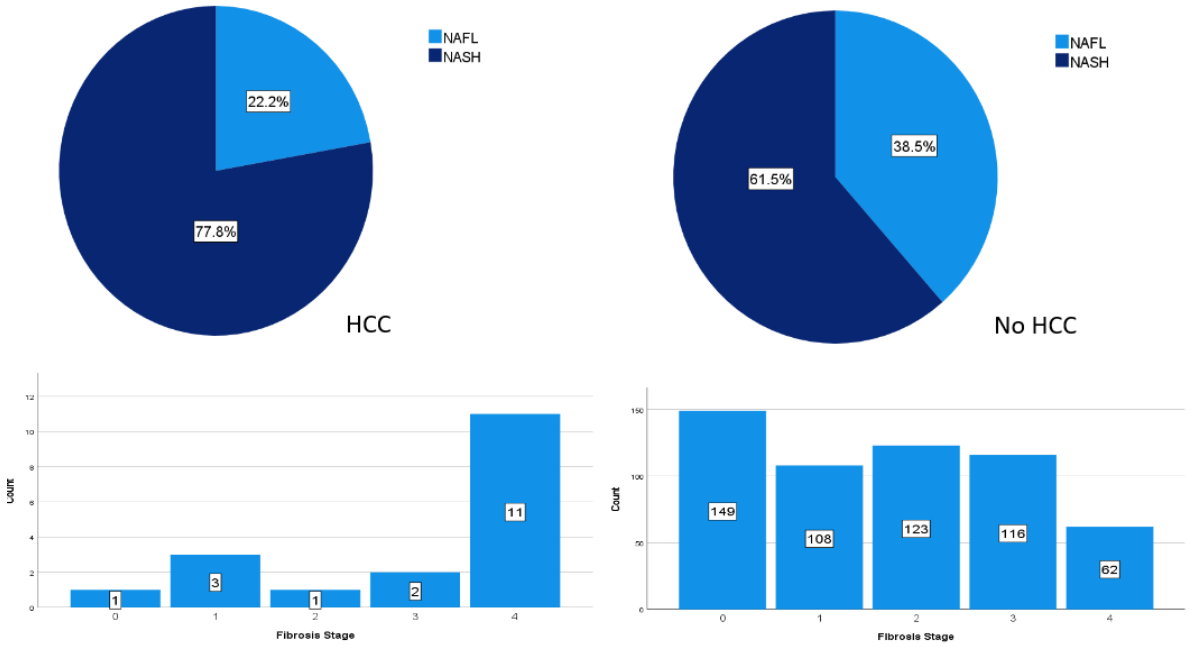
6.5.2 Comparison to those who did not develop HCC.

Comparison was made between the group of individuals who developed HCC and those who did not. It is important to note there is a significant difference in the group sizes (24 vs 578) and therefore the effect size of the differences may not be accurate, however clear trends in differences between the groups can be seen.

Patients who developed HCC were older (60 vs 54 years) at the time of inclusion to the study but there was no significant difference in BMI ($p=0.576$) or sex ($p=0.558$) between the groups. The group of patients with HCC had a higher GGT ($p=0.015$) and lower Albumin ($p<0.001$) and platelet levels ($p<0.001$) which is in keeping with more advanced liver disease in this group at baseline. The non-invasive scores were also higher for each tool in this group, which again would be in keeping with more advanced disease. Of note, the HCC group also had notably higher IgA levels ($p=0.012$) which has also been linked to been linked to more advanced disease. The incidence of IHD was also much higher in the group who developed HCC (46.2% vs 14.6%, $p=0.008$).

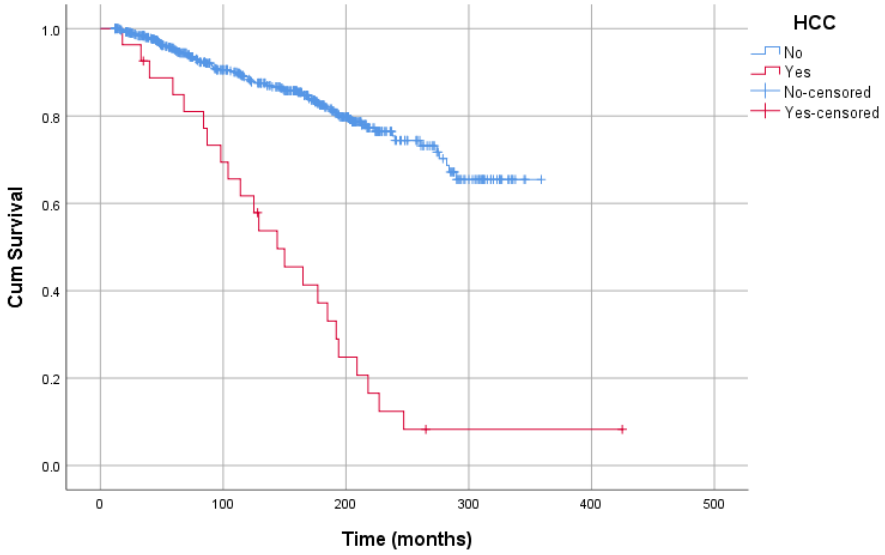
The baseline histology results were also compared between the two groups as shown in figure 6.8 below. The only notable difference between the groups is the higher proportion of advanced fibrosis (75.0% vs 31.7%, $p<0.001$) in the HCC group.

Figure 6.8. Comparison of baseline histology between HCC groups.



As may well be expected given the nature of hepatocellular carcinoma and the number of deaths in the HCC group already highlighted above, there was a significant difference in survival between these groups over time (Kaplan Meier log rank p<0.001). Figure 6.9 below displays the difference in survival by Kaplan Meier curves.

Figure 6.9. Kaplan Meier Survival curves stratified by the diagnosis of HCC. From the graph below it can be seen that those with HCC had a shorter survival period (p<0.001).



6.6 Liver transplantation

There were 10 patients from the Newcastle NAFLD cohort who underwent liver transplantation during the course of the study. All of these patients had cirrhosis at the time of the baseline event, accounting for 8.6% of those with cirrhosis. The indication for transplant in 55.6% (5) of the group was decompensation and poor synthetic function, while the remaining 44.4% (4) were listed for HCC. Almost two thirds (60%) of the transplant group were men, the mean age patients received the transplant was 59 ± 6 years, and the mean time to transplant was 3.6 ± 3.5 years (44 ± 42 months).

6.6.1 Baseline Characteristics

The liver transplant patients were further explored at the time of the baseline visit and the mean age at inclusion to the cohort was 56 ± 6 years old and 60% were men. The mean BMI for the group fell into the obese category (35.4kg/m^2) and the baseline mean liver laboratory results showed elevated ALT, AST and GGT levels. A baseline concurrent diagnosis of T2DM was present in 60% of the patients, and HTN was present in 50% of this cohort. Unfortunately, there were incomplete data available for the other clinical outcomes of interest (IHD and malignancy). The entire group (100%) cirrhosis, 40% diagnosed by histology and the remaining 60% were clinically diagnosed.

6.6.2 Final event characteristics

By the end of the follow-up period 50% (5) of those who received a liver transplant had died. The mean age at the final clinical event was 67 ± 8 years and the mean duration of follow-up was 10.3 ± 5.7 years (123 ± 68 months). ALT and GGT levels remained elevated, but AST levels had returned to within the normal range by the end of the study. As was seen across the entire Newcastle NAFLD cohort there was an increase in the presence of co-morbidities by the final event. Over half (55.6%, 5) had a new diagnosis of IHD, 57.1% (4) developed CKD and 50% (4) had been diagnosed with a Stroke.

Of the 5 patients that died during follow-up the mean age at death was 70 ± 7 years and time to death was 11.6 ± 5.3 years (140 ± 64 months). Three (60%) died of cardiovascular disease and the remaining 40% (2) died of sepsis.

6.6.3 Comparison to those who did not receive a transplant.

Differences between the group of patients those who were diagnosed with cirrhosis at baseline but did not have a transplant vs the group who did receive a liver transplant were

explored. At baseline the only significant difference between the two groups was Albumin levels in the transplant group were lower at baseline (38 vs 42 g/l, p=0.018) which is in keeping with the clinical indication for transplant in >50% of the group.

6.7 Chapter Summary

This chapter has explored liver specific outcomes of interest in the Newcastle NAFLD cohort. During the study a small group of 141 patients underwent repeat liver biopsies and of this cohort 64 (45.4%) were found to have disease progression (defined as a progression in fibrosis stage). Baseline presence of T2DM and a high steatosis score on the index biopsy were shown to be predictors for histological progression. Over the course of the follow-up there were 50 patients that went on to be newly diagnosed with cirrhosis: 30% were diagnosed histologically and the remaining patients were diagnosed radiologically or clinically. Baseline biopsy Steatosis and Fibrosis scores were found to predict this, but importantly the FIB-4 score was also found to be an independent predictor of clinical progression. Hepatocellular carcinoma occurred in 24 patients during the study, and was responsible for 12 deaths, and 10 patients received a liver transplant over the course of the follow-up.

6.8 Discussion

Throughout the literature the presence of advanced fibrosis has consistently been associated with poorer outcomes in patients with NAFLD.[97-100] Despite many studies exploring factors which may result in progression of fibrosis, the natural history is not well understood and there is significant heterogeneity seen. The Newcastle NAFLD cohort is one of the largest, single centre paired biopsy studies reported in the literature. Within this cohort it was found that a higher baseline steatosis score was associated with an increased risk of fibrosis progression with an odds ratio of 14.04 (95% CI 1.18 – 167.33, p=0.037).

This has also been observed in two historical paired biopsy studies[92, 93], and more recently Pais *et al*[95] identified steatosis as the only independent factor associated with disease progression in multivariate analysis (p=0.01). In contrast to this, Vilar-Gomez and colleagues[253] found that lower steatosis content was associated with worse survival and increased liver related events. However, this cohort only included patients with advanced

fibrosis (F3-4) at baseline and this low steatosis content being associated with poorer outcomes may be explained by the "burn-out" of hepatic fat seen in more advanced NAFLD fibrosis.

Further studies are needed to better understand the potential risk of disease progression with severe steatosis. In recent years there have been several using imaging techniques, such MRI-PDFF, as an alternative to liver biopsy as this modality is accurate in identifying hepatic steatosis content and fibrosis stage.[361]

In a study using MR-S to detect and measure hepatic steatosis and Fibroscan™ to assess liver stiffness, Lallukka and colleagues[362] found baseline liver fat content was the only independent predictor of increased liver stiffness assessed over 11.3 years (OR 2.17, 95% CI 1.05 – 4.46). Ajmera *et al*[363], in a study of 95 patients with well characterised NAFLD, demonstrated that patients with a higher liver fat content (defined as ≥15.7%) had a higher rate of fibrosis progression than those with a lower fat content (OR 6.67, 95% CI 1.01 – 44.1, p=0.049). More recently Tamaki and colleagues[364] also demonstrated that an MRI-PDFF response of ≥30% was an independent predictor for fibrosis regression (OR 6.46, 95% CI 1.10 – 37.0, p=0.04). This study potentially holds huge clinical significance as it is one of the first early phase clinical trials to demonstrate ≥1 stage fibrosis regression associated with improvement in hepatic fat content based on MRI findings. While MRI is not yet routinely available clinically this suggests that tools measuring steatosis, such as controlled attenuation parameter (CAP), could be useful monitor patients with NAFLD following a therapeutic intervention. However further research is required to validate alternative methods, such as CAP, and understand the long-term relationship with fibrosis changes.

Within the Newcastle NAFLD cohort, 23.4% of patients with "NAFL" at baseline had fibrosis progression over the follow-up period. The historical understanding that only patients with NASH would progress to advanced fibrosis has previously been disproven, and individuals with NAFL and metabolic risk factors such as obesity and T2DM are at risk of progression.[33, 94, 95] Of interest, within this group 73.3% had significant fibrosis stage progression over a short period of time and would best be described as "rapid progressors". The rate of fibrosis progression in NAFLD patients has been observed to be slower than other aetiologies of liver disease, Singh *et al*[45] found that patients with NASH progress 1 stage of fibrosis on average over 7 years and those with NAFL over 14 years. However, within this study it was also recognised that patients fell into two distinct categories: rapid or slow progressors. Similar to

the Newcastle cohort, Singh also found some of NAFL patients with progression over time were “rapid progressors”. Given the small numbers within this cohort they were unable to further explore features which may be associated with different rates of progression, and unfortunately the Newcastle NAFLD cohort had the same limitations. Further studies exploring the “rapid progressors” and features that predict this sub-cohort within the NAFLD population may provide important information in assessing and managing patients clinically.

Another finding of note from the Newcastle NAFLD cohort was the increased risk of clinical progression to cirrhosis seen with higher FIB-4 scores (OR: 7.04, 95% CI 1.64 – 30.17, p=0.009). Whilst in an ideal world a diagnosis of cirrhosis would be confirmed by histology, there are various reasons why this might not be possible, and the diagnosis is made clinically or from other investigations such as transient elastography. For this reason, there is a need to identify clinical parameters that predict patients at increased risk of developing cirrhosis using more “real world” approaches to research. One such example of a “real world” study used data from primary care databases in four European countries and included 136,703 patients diagnosed with NAFLD.[365] These patients were followed up until either a liver outcome (cirrhosis or HCC) was recorded or the end of the follow-up period and matched against “healthy” population controls. This study found that the incidence of a new diagnosis of cirrhosis was significantly higher in those with “high risk” FIB-4 scores at inclusion compared to those with “low-risk” scores (HR 33.24, 95% CI 8.82 – 125.34) in keeping with what was seen in the Newcastle NAFLD cohort.

Loomba *et al* have also recently undertaken a “real world” study utilising Medicare to screen a population of over 10 million people in the US for a diagnosis of NAFLD.[344] They identified 621,253 individuals who were then followed up over an 8-year period and liver outcomes recorded. In this study the presence of cardiovascular disease, CKD, dyslipidaemia, and diabetes were identified as independent predictors of disease progression, although this study did not explore non-invasive screening tools or blood results within the patients. Within the Newcastle cohort there was a trend to increased prevalence of HTN and IHD in those who progressed to cirrhosis over the follow-up but given the highly selective nature of this cohort the prevalence was increased across the entire cohort compared to a “healthy” population which will impact upon its use as an independent predictor for progression.

Other studies using paired biopsies have identified that the FIB-4 score was significantly higher in those who progressed compared to those who did not and therefore this has

potential to be used in the clinical setting to identify patients at greater risk of progression to cirrhosis.[33, 366] For example, patients with "high risk" FIB-4 but confirmed not to have cirrhosis on transient elastography or even liver biopsy, should likely have closer monitoring than those in the "low risk" category.

The incidence of HCC in the Newcastle NAFLD cohort, 4.1%, is in keeping with rates reported in other studies which describe a range between 2.4 – 12.8% in this population.[105, 106] Within the Newcastle cohort, mortality in these patients is reported as 52%. HCC is still reported as one of the leading cancer causes of death worldwide. Historically the most common aetiology for HCC was chronic viral hepatitis (Hepatitis B and C). However, despite rates of HBV and HCV declining globally due to the advent of DAA therapies, the associated mortality from HCC in western countries has continued to increase.[367] One study demonstrated the USA had an increase in HCC mortality of 35% between 2002-2012, which was related to the increasing incidence of NAFLD related HCC.[368, 369] Studies have shown a 9% annual increase in cases of NAFLD-HCC[37] and a 7.7-fold rise in the proportion of patients listed for liver transplantation.[343] The prognosis for patients with NASH-HCC is poor for reasons including advanced age at diagnosis, the presence of significant co-morbidities such as cardiovascular disease and late detection which often means there are no viable treatment options. One of the reasons for late detection in this group is likely to be inadequate screening in the NAFLD group where HCC can occur in the absence of cirrhosis. 20-30% of cases occur in patients with F3 fibrosis[105, 106] and current U.K. guidelines support screening only in those with established cirrhosis. It is therefore important to explore other factors predicting HCC development and while only a small number of patients in the Newcastle cohort developed HCC there was a trend to significantly higher FIB-4 scores in this group which has also been reported by Kanwal *et al*, even in those without established cirrhosis.[370] Further studies into the use of non-invasive tools to predict HCC could provide a cost-effective method of rationalising screening in patients with F3. The presence of cardiovascular disease was also significantly higher in those who developed HCC in the Newcastle cohort (p=0.002). Given the association between CVD and NAFLD, and the shared risk factors for the development of progressive disease, including oxidative stress and low-grade inflammation, it is unsurprising there is a link between the presence of CVD and HCC.[27, 239, 371] However, the association is not well understood and upon reviewing the existing literature there appears to be a paucity of evidence exploring the relationship

between cardiovascular disease and the development of HCC in NAFLD patients. Further research into this may also provide an additional strategy in assessing the risk and implementing screening for HCC, particularly in those with F3.

As recognised in chapter 5, this study has several limitations including the small numbers of patients with liver outcomes such as HCC or liver transplant. The above chapter explores these liver outcomes of interest within the Newcastle cohort and found several factors associated with histological progression, such as baseline steatosis grade, which may warrant further research in order to better understand those individuals at risk of progressive disease, particularly “rapid progressors”. The FIB-4 score has also been identified as a potential tool to screen for those at risk of developing HCC and again would warrant further research.

A small sub-cohort of the Newcastle NAFLD group, historically used in the validation of the ELF panel, will be further explored in the following chapter.

Chapter 7: Enhanced Liver Fibrosis (ELF) Panel

7.1 Introduction

Non-alcoholic fatty liver disease (NAFLD), over the last decade, has become the most common cause of chronic liver disease worldwide [2, 50]. Studies have shown that the most significant predictor of outcome is fibrosis stage and liver biopsy remains the gold standard investigation to evaluate this[45, 101, 253]. However, with the increasing prevalence of NAFLD, obtaining a liver biopsy on all patients is neither possible nor appropriate given it is associated with significant risks and is itself an imperfect investigation subject to sampling errors and intra-observer variability.

As an alternative to liver biopsy, several blood-based biomarkers have been developed in an attempt to stage liver disease non-invasively. The Original European Liver Fibrosis panel (OELF) was initially developed to detect fibrosis using easily obtained blood samples and clinical parameters. The panel consisted of 3 blood markers: type III procollagen peptide (PIIINP), hyaluronic acid (HA), and tissue inhibitor of metalloproteinase-1 (TIMP1), and age.[372]

This algorithm was subsequently simplified by Guha *et al*[170] in 2008 using a cohort of 192 subjects from 2 tertiary hepatology centres in the U.K.: Nottingham and Newcastle hospitals. Guha reported no reduction in diagnostic performance by removing age from the OELF formula, and the Enhanced Liver Fibrosis panel (ELF) has been shown to accurately exclude the presence of advanced fibrosis in patients with NAFLD. In 2010 Parkes *et al* also demonstrated this simplified ELF panel performed well in all chronic liver disease in a follow-up study of the original cohort.[373]

The 2016 National Institute of health and Care Excellence (NICE) guideline on NAFLD has recommended the use of the ELF panel to assist in the diagnosis of advanced fibrosis, particularly in a primary care setting, and aide triage to secondary care.[169] The panel was developed with the aim of identifying fibrosis and performs reasonably well in distinguishing advanced fibrosis/cirrhosis (F3/4) from F0-2.[170] Given fibrosis stage has been reported as a predictor of outcomes the ELF panel is likely to also predict liver related outcomes. In this chapter, the clinical outcomes of the Newcastle patients included in the 2008 paper will be explored and the ability of the ELF panel to predict outcomes will be assessed. The

performance of the ELF panel will also be compared to other commonly used non-invasive tools such as the FIB-4 score and NFS.

7.2 Chapter Aims

This chapter will:

1. Describe baseline and follow-up characteristics of the sub-cohort of the Newcastle NAFLD patients who were involved in the 2008 ELF study.
2. Explore the ability of ELF, and its individual components to predict mortality.
3. Compare ELF to other non-invasive scores in its ability to predict mortality.

7.3 Methods

7.3.1 Patient selection at baseline

At the time of the original study (2008), 104 patients were recruited from the Newcastle upon Tyne Hospitals based on the following criteria:

1) elevated aminotransferases (AST or ALT)
2) appropriate exclusion of liver disease of other origin including alcohol induced or drug induced liver disease, autoimmune or viral hepatitis, or cholestatic or metabolic/genetic liver disease.

Other causes of liver disease were excluded using specific clinical, biochemical, radiographic, or histological criteria, and patients were examined for a history of potentially harmful alcohol consumption.

Participants underwent a liver biopsy to confirm the clinical diagnosis and stage disease between October 2002 and December 2006. Blood samples were also taken within 3 months of the biopsies and included serum levels of TIMP-1, HA and P3NP; the individual components of the ELF panel. These ELF components were processed at an independent reference laboratory; iQur Limited, Southampton.

7.3.2 Patient selection at follow-up

In 2020, as part of the study being undertaken into the long-term outcomes of NAFLD, the patients who had taken part in the original ELF derivation study in 2008 were reviewed for a follow-up event.

At this time 95 patients were eligible to take part; 9 were excluded because of a change in clinical condition including the development of harmful alcohol consumption, use of steatogenic drugs such as Tamoxifen, and subsequent diagnosis with an alternative aetiology such as autoimmune liver disease.

Of the 95 eligible participants 26% (25) had consented to taking part in the European NAFLD Registry and the remaining 74% had follow-up data collected in keeping with CAG approvals, as outlined in chapter 2.

The results discussed in this chapter will only reflect the Newcastle ELF participants, not the entire cohort, and analysis will be performed on the 95 patients that underwent follow-up.

7.3.3 Data management

The same principles of data management were used for the ELF cohort as for the entirety of the Newcastle NAFLD cohort, as documented in chapter 2. Data were collected using the existing REC, CAG and HRA approvals, and was recorded in the European NAFLD Registry. The same approach was taken regarding missing data points, given the original ELF study took place in 2008 there were anticipated gaps in the available data, which are acknowledged throughout. No imputation of missing data points were made. Data monitoring was also managed as per the overall cohort, utilising the monitoring processes in place for the European NAFLD Registry.[324]

7.3.4 ELF Panel Update

Following the introduction of the Enhanced Liver Fibrosis panel by Guha *et al*[170] in 2008 the algorithm as it is clinically used has undergone a lab-based revision and had various differing cut-offs proposed for its clinical use.

In 2009 Nobili *et al*[374] very simply added 10 to the Guha score in order to generate only positive scores which would reduce potential transcription errors in clinical use.

The laboratory technology used to generate the ELF panel results was updated in 2011 by Siemens to utilise a Centaur analyser. At this time the creators took particular care to ensure the results generated would be identical to the original assays analysed thus enabling the ongoing use of the same algorithm[375].

There have been various studies proposing different cut-off scores in order to optimise the performance of the test, and importantly, although sometimes overlooked, the manufacturer also recommends specific thresholds to be contemplated when excluding advanced fibrosis. These cut-offs (<9.8 and ≥11.3) were approved by the FDA for clinical use in 2021.

In contrast to the threshold approved by regulators, in 2016 NICE guidelines[169] recommended the use of ≥10.51 to diagnose advanced liver fibrosis based in large part on Nobili *et al* findings (AUROC 0.99, PPV 80%, NPV 100%). It is important to note however that this study used a paediatric cohort which has resulted in some controversy surrounding its use and there remains significant debate as to the optimal cut-offs to use.[146, 376-378]

As one of the objectives of this chapter is to review the effectiveness of the clinical application of the ELF panel results analysis will be performed using the current NICE guideline algorithm to ensure valid comparison.

7.3.5 Statistical Analysis

The principals of data analysis outlined in the methodology chapter (2) were utilised in the analysis of the Newcastle ELF sub-cohort. Area under receiver operating curves were generated to compare the original performance of the ELF panel to the result of the FIB-4 and NFS scores. The data were found to be non-parametric and therefore Mann-Whitney U tests were used to establish any differences when comparing groups. Binary logistic regression was undertaken to explore differences in progression and Kaplan Meier survival curves and Cox regression modelling was used to explore mortality outcomes.

7.4 Results

7.4.1 Baseline Characteristics

Almost two thirds (62%) of the ELF cohort were male and 94% Caucasian. The mean age at the time of the original study was 47 ± 13 years old and the median BMI fell into the obese

category (32.5kg/m^2). A fifth of patients had a diagnosis of T2DM at the time of the baseline visit and 11.6% had cirrhosis. The mean liver related laboratory results showed elevated blood enzymes: ALT, AST and GGT, in keeping with common blood abnormalities seen in NAFLD. Table 7.1 below displays the baseline characteristics of the ELF cohort.

Table 7.1. Baseline characteristics of ELF cohort. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

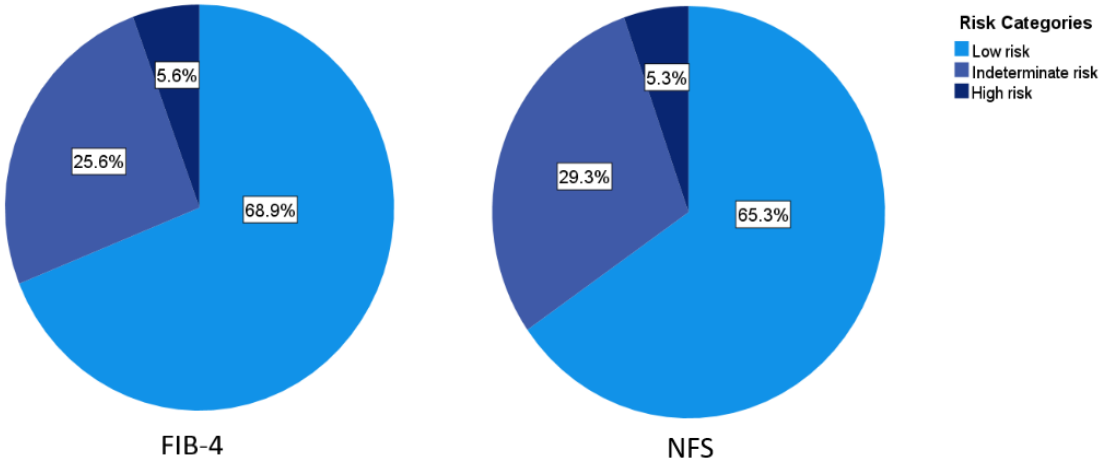
Variable	n	Result
Age (years)	95	47 ± 13
Sex (male)	95	59 (62.1%)
BMI (kg/m^2)	91	32.5 (23.4-46.5)
ALT (0 – 40) [U/l)	93	66 (13-247)
AST (0 – 40) [U/l)	92	40 (17-218)
GGT (0 – 70) [U/l)	91	79 (16-363)
Albumin (35 – 50) [g/l]	93	45 ± 4
Platelets (150 – 450) [x10^9/l]	93	244 ± 66
PT (10 – 13) [seconds]	67	12 ± 1
IgA (0.8 – 3.0) [g/l]	89	2.75 (0.07-12.90)
T2DM	94	21 (22.3%)
HTN	73	24 (31.5%)
MetS	65	41 (60.0%)
IHD	63	8 (12.7%)
Malignancy	70	1 (1.4%)
Cirrhosis	95	11 (11.6%)

The mean results of the non-invasive scores are documented in table 7.2 below. The majority of patients fell into the “low risk” categories for the non-invasive tools as can be seen in figure 7.1.

Table 7.2. Baseline non-invasive tool results of ELF cohort.

Non-invasive Tool	n	Result
FIB-4 Score	90	1.05 (0.25-8.10)
NFS	75	-2.07 (-5.14-4.93)

Figure 7.1. Proportion of patients in each risk category of non-invasive tools for ELF cohort at baseline. Cut offs used were FIB-4 1.30 and 2.67, and NFS -1.455 and 0.676.



The mean results of the ELF panel are displayed below in table 7.3. For the remainder of this chapter any results pertaining to the "ELF score" will refer to Nobili *et al*[374] results which will allow for clinical interpretation based upon the current NICE guidelines.

Table 7.3. Median (range) results of ELF panel: individual components and calculated score.

ELF Panel	n	Result
TIMP-1	95	765.43 (483.34-1602.06)
HA	95	26.80 (3.17-607.58)
P3NP	95	8.60 (4.37-40.56)
ELF score [Guha [170]]	95	-0.29 (-2.19-2.67)
ELF score [Nobili [374]]	95	9.71 (7.81-12.67)

Ninety two of the ninety five patients included in the Newcastle ELF cohort in 2008 had biopsy results still available to be reviewed at this time of this review. The remaining 3 were known to have cirrhosis, diagnosed either clinically or from incomplete biopsy results (steatosis, ballooning scores etc no longer available).

Almost half (42.2%) of the ELF cohort had NASH present on baseline histology, with 13% having advanced fibrosis, as defined by F3-4 histologically. Table 7.4 below shows a breakdown of the histological features seen on at baseline for the Newcastle ELF cohort.

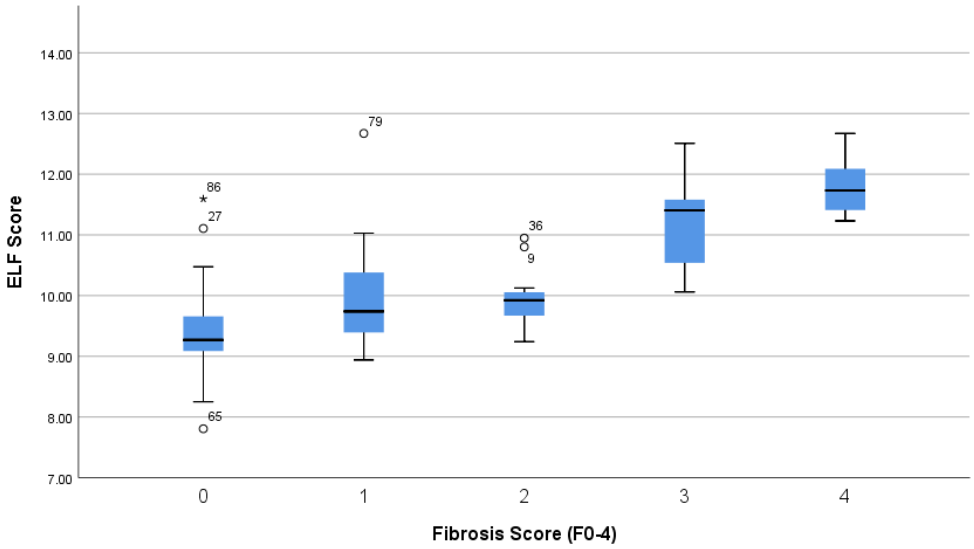
Table 7.4. Baseline histology of ELF cohort. "Not significant" fibrosis was defined as F0-1, "clinically significant" ≥F2 and "advanced" F3-4. Fibrosing steatohepatitis was defined as patients with a NAS score ≥4 and ≥F2.

Variable	n	Result
Steatosis • 0 • 1 • 2 • 3	92	 0 31 (33.7%) 46 (50.0%) 15 (16.3%)
Ballooning • 0 • 1 • 2	92	 52 (56.5%) 31 (33.7%) 9 (9.8%)
Inflammation • 0 • 1 • 2 • 3	92	 0 41 (44.6%) 39 (42.4%) 12 (13.0%)
Fibrosis • F0 • F1 • F2 • F3 • F4	92	 43 (46.7%) 25 (27.2%) 12 (13.0%) 6 (6.5%) 6 (6.5%)
Fibrosis category • No significant (0-1) • Clinically significant (2-4) • Advanced (3-4)	92	 68 (73.9%) 24 (26.1%) 12 (13.0%)
NASH (present)	92	39 (42.4%)
NAS Score Categories • 1-2 • 3-4 • ≥5	92	 39 (42.4%) 36 (39.1%) 17 (18.5%)
SAF Activity Score	92	1 ± 1
Fibrosing steatohepatitis	92	19 (20.7%)

7.4.2. Original performance of ELF panel in Newcastle Cohort.

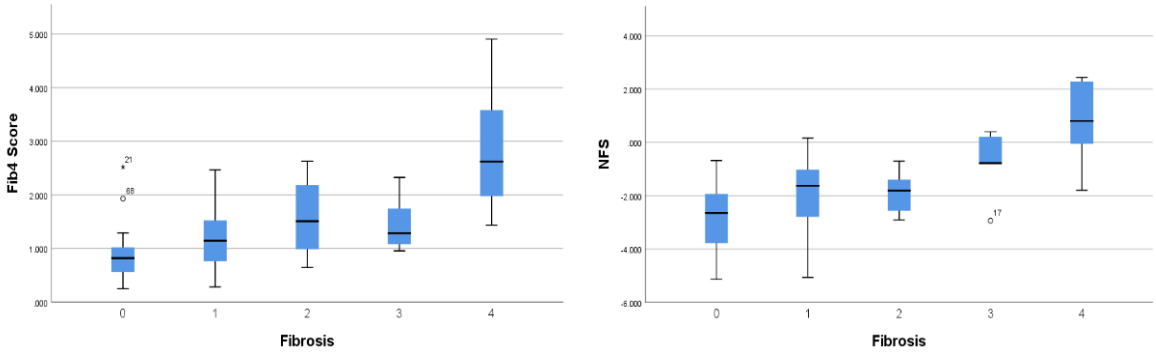
The original performance of the ELF panel in identifying advanced fibrosis was re-examined. Figure 7.2 below shows the distribution of the ELF results corresponding to histologically confirmed fibrosis stage and clearly demonstrates a relationship between increasing ELF result and increasing stage of fibrosis.

Figure 7.2 Boxplot illustrating distribution of ELF results related to histological fibrosis stage at baseline.



As the aim of this chapter is to compare the performance of the ELF panel against the other commonly used non-invasive tools, the FIB-4 score and NFS, similar boxplots were created for these results. As can be seen in figure 7.3 below, the distribution of these non-invasive tool results corresponding to the histology is similar to the ELF panel above.

Figure 7.3. Boxplots of FIB-4 and NFS results correlating to histological fibrosis stage at baseline.



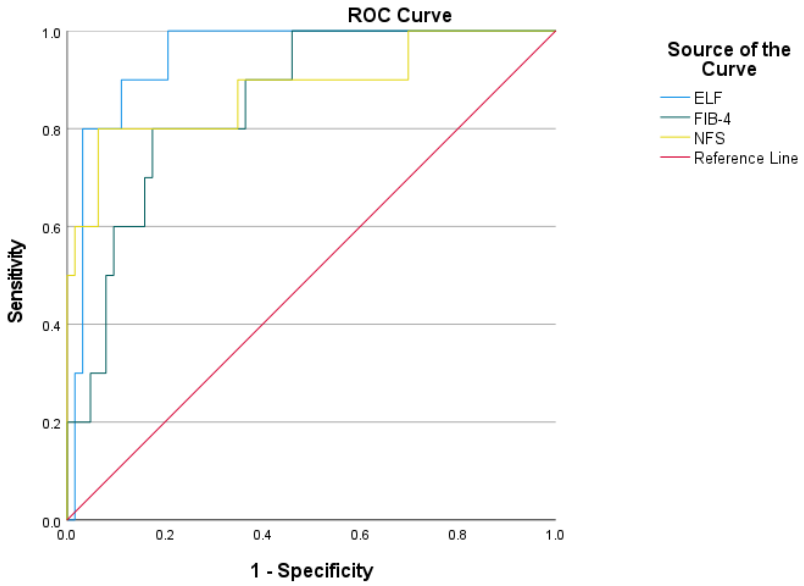
Area under receiver operating curves were also generated for each tool to assess the performance in identifying advanced fibrosis (F3-4). Each non-invasive tool performed well,

with overlapping confidence intervals suggesting no score was superior as can be seen from table 7.5 and figure 7.4

Table 7.5. AUROC results for ELF, FIB-4 and NFS demonstrating performance in predicting advanced fibrosis (F3-4) in the ELF cohort.

Non-invasive tool	Cut-off	AUROC (95% CI)	p value	Sensitivity*	Specificity*
ELF	10.51	0.954 (0.91-1.00)	<0.001	91.7%	87.5%
FIB-4	2.67	0.824 (0.72-0.93)	<0.001	25.0%	98.7%
NFS	0.676	0.881 (0.74-1.00)	<0.001	30.0%	100%

Figure 7.4. ROC curves demonstrating the performance of ELF, FIB-4 and NFS predicting advanced fibrosis (F3-4). This shows the ELF performed best with AUROC 0.954.

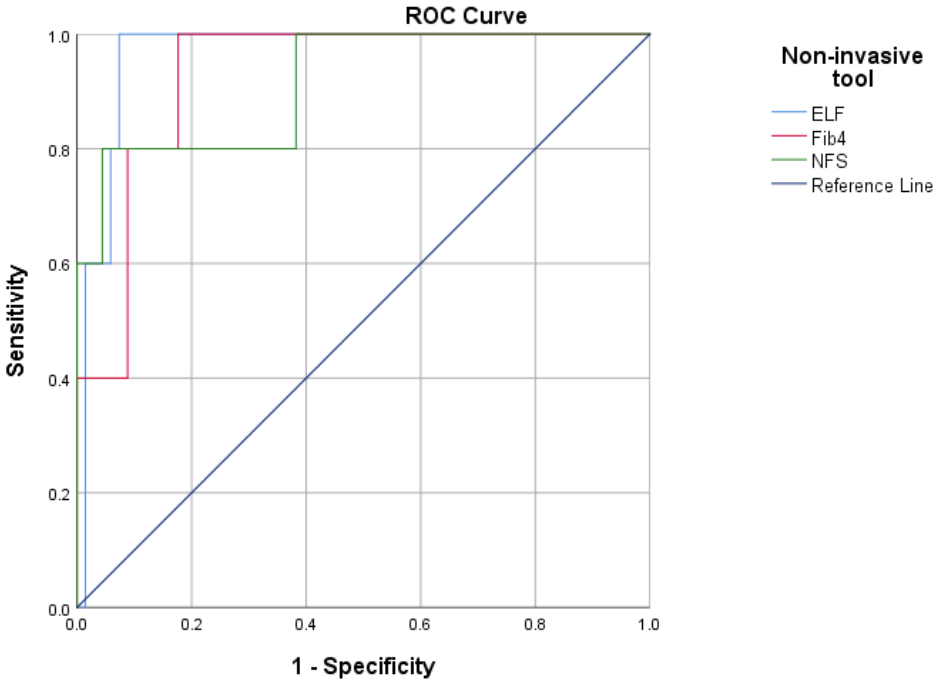


These scores performances were also tested in identifying F4 and again each non-invasive test performed well.

Table 7.6 AUROC results for ELF panel, FIB-4 and NFS demonstrating performance in predicting F4 in the ELF cohort.

Non-invasive tool	Cut-off	AUROC (95% CI)	p value	Sensitivity*	Specificity*
ELF	10.51	0.966 (0.93 – 1.00)	<0.001	100%	82.6%
FIB-4	2.67	0.920 (0.81 – 0.99)	0.001	50%	98.8%
NFS	0.676	0.915 (0.78 – 1.00)	0.002	60.0%	96.5%

Figure 7.5. ROC curves demonstrating the performance of ELF panel, FIB-4 and NFS predicting F4. From the curves ELF can be seen to have performed best with AUROC 0.966.



7.4.3 Follow-up characteristics

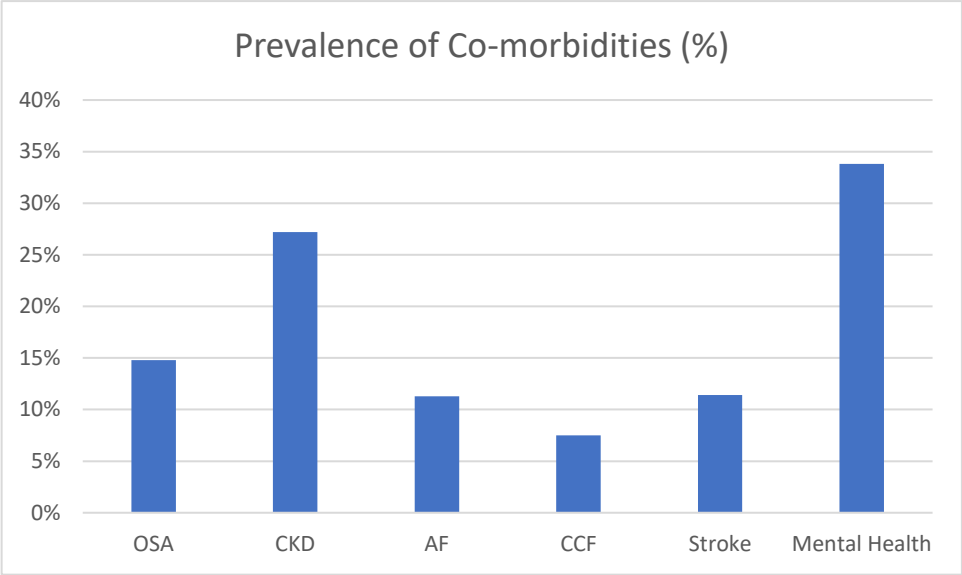
At the time of follow-up 19 (18.9%) of the original cohort had died, 77 (81.1%) were still alive. The mean age at the final follow-up event was 66 ± 14 years and the median duration of follow-up was 17.8 years (3.3-35.4). The mean BMI remained in the obese category (35.0 kg/m^2) and the proportion of patients with T2 DM more than doubled in the follow-up period to 58% (51). The majority of mean liver laboratory results had returned to within normal limits by follow-up, however the GGT remained elevated. The results of these results are displayed in table 7.6 below.

Table 7.7. Follow-up characteristics of ELF cohort. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	n	Results
Age (years)	95	66 ± 14
BMI (kg/m^2)	9	35.0 ± 4.24
ALT (0 – 40) [U/l)	75	33 (8-136)
AST (0 – 40) [U/l)	17	35 ± 12
GGT (0 – 70) [U/l)	22	63 (29-679)
Albumin (35 – 50) [g/l]	76	44 (25-52)
Platelets (150 – 450) [x10^9/l]	74	226 (24-648)
PT (10 – 13) [seconds]	34	12 (11-41)
IgA (0.8 – 3.0) [g/l]	2	2.84 ± 0.79
FIB-4	17	1.83 (0.51-5.30)
NFS	3	-0.83 ± 0.31
T2DM	88	51 (58.0%)
HTN	90	65 (72.2%)
MetS	53	43 (81.1%)
IHD	83	18 (21.7%)
Malignancy	87	20 (23.0%)
Cirrhosis	87	21 (24.1%)

As with the main Newcastle cohort, the ELF sub-cohort had an increased prevalence of several other co-morbidities present at the end of follow-up including CKD (27.2%), OSA (14.8%) and Stroke (11.4%). Figure 7.6 below shows the prevalence of the “other” co-morbidities by the end of follow-up. 8 participants (8.4%) were diagnosed with HCC during the follow-up.

Figure 7.6. Prevalence of other co-morbidities at follow-up.



OSA=obstructive sleep apnoea, CKD=chronic kidney disease, AF=atrial fibrillation, CCF=congestive cardiac failure.

7.4.4. Death

As detailed above, during the follow-up of the study 18 (18.9%) of the Newcastle ELF cohort died. The mean age at time of death was 75 ± 12 years old and the mean time to death was 11.8 ± 5.5 years (141 ± 66 months). Eight (44.4%) of those who died were men.

The cause of death was recorded in the same way as the entire Newcastle cohort, using the details obtained from death certificates and medical records. Details of the causes of death for the Newcastle ELF cohort are recorded in table 7.7 below.

Table 7.8. Causes of death in the ELF cohort.

Cause of Death	Number of Patients (n=18)
Liver related • Cirrhosis • HCC	8 (44.5%) • 3 (16.7%) • 5 (27.8%)
Cardiovascular	2 (11.1%)
Extrahepatic Malignancy	3 (16.7%)
Other	5 (27.8%)

Of those who died of “malignancy”: one died of Colon cancer, one Gastric cancer and the third from small cell lung cancer. The “other” causes of death included respiratory disease, infection, frailty, and renal failure.

7.5 ELF and all-cause mortality.

The relationship between ELF results and all-cause mortality was then explored. A comparison of individual ELF panel results and the overall ELF score was made between those alive vs dead. The baseline mean result of the complete ELF score in those that had died was significantly higher than those who lived, 11.38 vs 9.70 ($p<0.001$), and this was also true for each individual component of the panel. Table 7.8 below demonstrates these results.

Table 7.9. Comparison of baseline ELF results between alive and all-cause mortality groups. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Alive n= 77	Dead n= 18	p value
TIMP-1	743.41 (489.66-1268.28)	975.07 ± 308.02	0.004*
HA	25.08 (3.17-294.98)	105.28 (13.59-607.58)	<0.001*
P3NP	8.34 (4.37-40.56)	13.79 ± 5.88	0.008*
ELF panel	9.70 ± 0.79	11.38 (8.88-12.67)	<0.001*
*Mann Whitney U test			

When comparing the result of FIB-4 and NFS in these groups there were similar findings with significantly higher results in the all-cause mortality group ($p<0.001$ for both). Table 7.9 below displays the result of these non-invasive tools.

Table 7.10 Comparison of baseline FIB-4 and NFS results between all-cause mortality groups. Normally distributed data is displaced as mean ± standard deviation, non-parametric data as median and range.

Variable	Alive n= 77	Dead n= 18	p value
FIB-4	0.94 (0.25-2.63)	1.92 (0.68-8.10)	<0.001*
NFS	-2.50 ± 1.29	-0.71 (-2.08-4.93)	<0.001*
*Mann Whitney U test			

Kaplan Meier survival curves were generated to explore the different categories of the ELF panel, FIB-4 score and NFS. The ELF results were grouped into <9.80 as "low-risk", 9.80 – 10.50 as "indeterminate risk" and ≥10.51 as "high-risk", the FIB-4 and NFS category cut-off were as in previous chapters. Figures 7.6 – 7.8 below demonstrate these survival curves.

Figure 7.7. Kaplan Meier survival curves stratified by baseline ELF risk categories in all-cause mortality. Using the cut offs <9.80 as "low risk", 9.80-10.50 as "indeterminate risk" and ≥10.51 as "high risk" it can be seen that those in the high risk group have an increased risk of all-cause mortality.

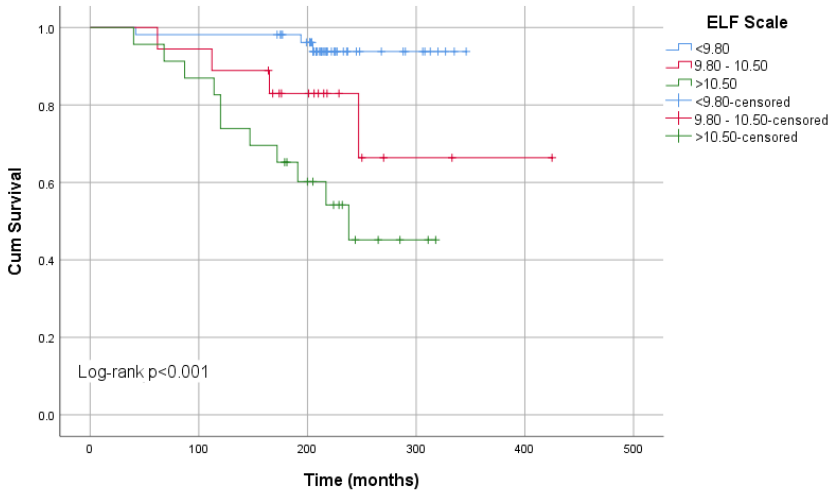


Figure 7.8. Kaplan Meier survival curves stratified by baseline FIB-4 risk categories in all-cause mortality. Cut-offs used were <1.30 "low risk", 1.30-2.67 "indeterminate risk" and >2.67 "high risk". From the graph it can be seen that those in the "high-risk" group had a significantly increased risk of mortality (p<0.001) and shorter survival time than those in the "low-risk" group.

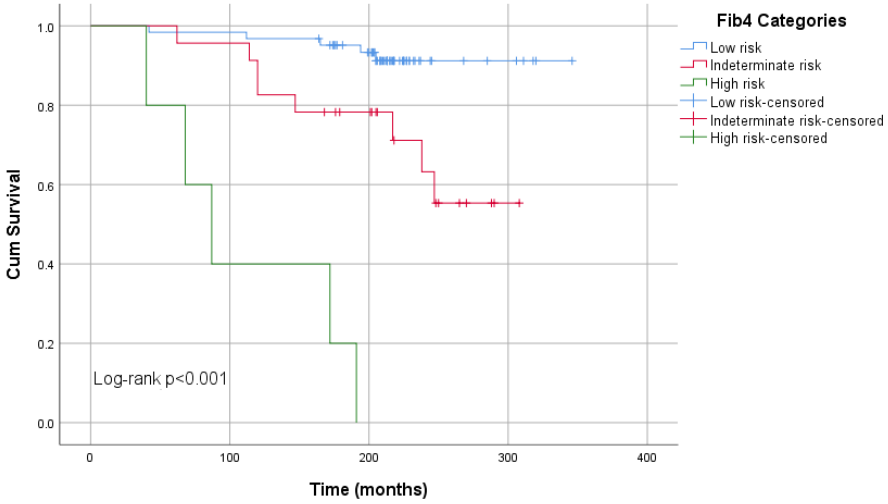
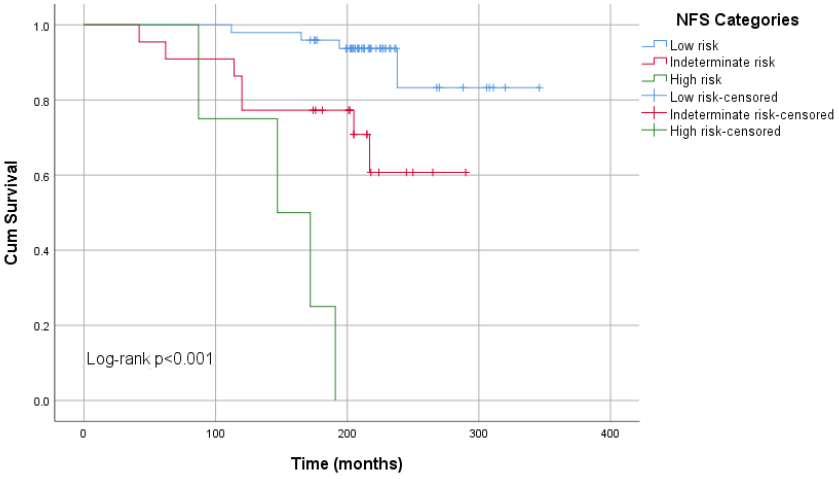


Figure 7.9. Kaplan Meier survival curves stratified by baseline NFS risk categories in all-cause mortality. Cut-offs used were <-1.455 "low risk", -1.455-0.676 "indeterminate risk" and >0.676 "high risk". From the graph it can be seen that those in the "high-risk" group had a significantly increased risk of mortality (p<0.001) and shorter survival time than those in the "low-risk" group.



A clear difference in survival between the "low risk" and "high risk" categories can be seen for each of the three tools used (p<0.001 for each).

Univariate Cox Regression modelling was then undertaken for each individual component of the ELF panel, the complete ELF result, and the FIB-4 and NFS scores to further explore any. The ELF panel had the biggest HR of all these at 2.80 (95% CI 1.87 – 4.19, p<0.001), the results of the univariate Cox Regression are shown in table 7.10 below.

Table 7.11. Cox Regression hazard ratios of non-invasive tools and ELF panel predicting all-cause mortality. Each individual component of the ELF panel, the combined ELF result, and the FIB-4 and NFS scores were each entered into a Cox regression model and found to be predictors of all-cause mortality. Hazard ratios generated are shown in the table below.

Variable	HR	95% CI	p
TIMP-1	1.003	1.002 – 1.005	<0.001
HA	1.008	1.005 – 1.010	<0.001
P3NP	1.06	1.004 – 1.11	0.034
ELF panel	2.80	1.87 – 4.19	<0.001
FIB-4	1.56	1.29 – 1.89	<0.001
NFS	1.63	1.35 – 1.97	<0.001

Proportionality of the risk categories for the ELF panel, FIB-4 and NFS risk categories were assessed prior to using them in a Cox model also. The ELF panel was the only result found to be non-proportional, which may result in an overestimation of risk with time. When comparing the “low risk” to “high risk” categories of each score there was an increased risk of mortality in the higher risk group. The ELF panel “high risk” category was associated with a hazard ratio of 10.43 (95% CI 2.90 – 37.50, p<0.001), and after adjusting for age it remained a significant predictor of all-cause mortality with aHR 5.58 (95% CI 1.59-21.46, p=0.008). The results of the other scores can be seen in table 7.11 below.

Table 7.12 Cox Regression hazard ratios for non-invasive scores predicting all-cause mortality. Using "low-risk" as the comparator reference for each score, and the cut-offs as described throughout (ELF <9.80, 9.80-10.50, ≥10.51. FIB-4 <1.30, 1.30-2.67, >2.67. NFS <-1.455, -1.455-0.676, >0.676.), each tool was examined for its prognostic abilities in all-cause mortality and hazard ratios generated can be seen in the table below.

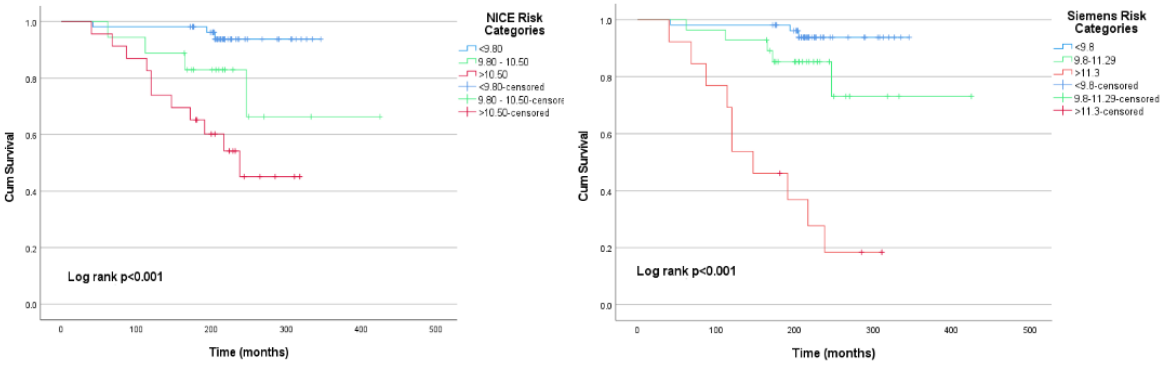
Variable	HR (95% CI)	aHR (95% CI)*
ELF panel • Low (ref) • Indeterminate • High	 4.43 (0.99-19.85), p=0.052 10.43 (2.90-37.50), p<0.001	 2.25 (0.49-10.40), p=0.300 5.85 (1.59-21.46), p=0.008
FIB-4 Score • Low (ref) • Indeterminate • High	 4.09 (1.31-12.74), p=0.015 35.90 (9.65-133.62), p<0.001	 1.95 (0.51-7.45), p=0.330 16.04 (3.46-74.33), p<0.001
NFS • Low (ref) • Indeterminate • High	 4.74 (1.38-16.22), p=0.013 28.07 (6.42-122.75), p<0.001	 1.74 (0.34-9.02), p=0.511 9.38 (1.41-62.35), p=0.021
*Adjusted for age		

7.6 Comparison of ELF cut-offs

As described above, the recommended NICE cut-off of ≥10.51 differs from that of the regulatory approved ≥11.3 as recommended by the manufacturer of the ELF panel. A comparison of the prognostic performance of ELF using both these cut-offs was therefore undertaken.

There were fewer patients that fell into the "high risk" category when using the ≥11.3 cut-off, 13 (13.7%) vs 23 (24.2%). Figure 7.10 below demonstrates the difference in Kaplan Meier survival curves using the differing cut-offs.

*Figure 7.10. Kaplan Meier survival curves for all-cause mortality stratified by baseline ELF risk categories for NICE (<9.80, 9.80-10.50, ≥10.51) and Siemen's (<9.80, 9.80-11.29, **≥11.30)** cut-offs. From the graphs below it can be seen the Siemen's high risk category may perform better in identifying those at high risk of mortality than the NICE recommended cut off.*



Hazard ratios for the risk categories were also generated using Cox Regression analysis as above and using the regulatory approved cut-off of ≥11.3 as "high risk" showed significant prognostic value in predicting all-cause mortality, HR 20.92 (95% CI 5.70-76.76, p <0.001). Table 7.13 below shows the comparison of the different cut-offs.

*Table 7.13 Cox Regression hazard ratios for ELF risk categories predicting all-cause mortality. Using "low-risk" as the comparator reference the NICE (<9.80, 9.80-10.50, ≥10.51) and Siemen's (<9.80, 9.80-11.29, **≥11.30)** risk categories were entered into a Cox regression model to assess their ability to predict mortality. Hazard ratios generated as shown in the table.*

Variable	HR (95% CI)
NICE ELF cut-offs • Low (ref) • Indeterminate • High	 - 4.43 (0.99-19.85), p=0.052 10.43 (2.90-37.50), p<0.001
Siemens ELF cut-offs • Low (ref) • Indeterminate • High	 - 3.43 (0.82-14.38), p=0.092 20.92 (5.70-76.76), p<0.001

7.7 Discussion

Validation of the Enhanced Liver Fibrosis (ELF) panel was originally detailed by Guha and colleague in 2008.[170] This study involved 192 patients, which included 104 from the Newcastle NAFLD cohort, and demonstrated that the ELF score could accurately identify the presence of advanced fibrosis (F3-4) with an AUC of 0.90. It is therefore unsurprising that upon reviewing this same cohort, with some of the original patients excluded as detailed

above, that these results continue to show excellent performance of the ELF panel in identifying cirrhosis with an AUROC 0.954 (95% CI 0.91-1.00, p<0.01).

In this chapter a comparison to the other non-invasive tools was undertaken which was not reported in the 2008 paper, and this demonstrated that the ELF score outperformed both the FIB-4 (AUC 0.920) and NFS (AUC 0.915), with higher sensitivity and specificity. Across the literature there are not many head-to-head studies comparing the use of ELF to other non-invasive scores in a NAFLD specific cohort, but the Newcastle cohort findings are in keeping with those reported from the STELLAR trials which found that the ELF score performed best of these tools (AUROCs ELF 0.80, FIB-4 0.78, NFS 0.74).[171] Staufer and colleagues[379] also recently demonstrated that the ELF panel outperformed the FIB-4 and NFS in a study of 186 histologically confirmed NAFLD patients comparing six non-invasive methods of advanced fibrosis detection. (AUC 0.90 vs 0.81 vs 0.79 respectively). However, there remains debate as to the optimum cut-offs used for defining "advanced fibrosis" with many studies citing thresholds which differ from regulatory approved cut-offs.

Within the Newcastle ELF cohort there was a notably high proportion of deaths due to HCC when compared to the overall cohort (27.8% vs 12.5%). Numbers were significantly smaller in this sub-cohort, which limits the impact of any further exploration of this. However, the mean age of death in the ELF sub-cohort was significantly older than the complete cohort (75yrs vs 64yrs) which is likely one of the simplest explanations for this finding given increasing age is an established risk factor for the development of HCC. This group of patients were also recruited prior to 2008 and therefore the follow-up period is likely to be longer, which will again increase the risk of the development of liver end points such as cirrhosis and HCC.

One of the key questions to be explored by this study of the ELF panel was the potential for its use in the prediction of mortality in NAFLD patients. The Newcastle ELF cohort demonstrated a clear increased risk of all-cause mortality in those who fell into the "high risk" category of ELF score (defined as ≥10.51) compared to those in the "low risk" category. After adjusting for age those who were in the "high risk" category had an aHR 5.85 (1.59-21.46), p=0.008. Within the literature there are few studies which have explored the use of ELF in predicting mortality outcomes in NAFLD cohorts. Irvine *et al*[380] recently demonstrated in an all-cause liver disease cohort that the ELF score was an independent risk factor for the development of liver outcomes including decompensation and HCC (aHR 2.53,

95% CI 1.45 – 4.43). Parkes and colleagues[373] similarly found those with "high" ELF categorical scores (defined as 12.52-16.67 in this study) were associated with adjusted hazard ratios 75.7 (95% CI 17.6-325.4). This study again was an all-aetiology liver disease study and adjustments were made for age, sex, alcohol, smoking, aetiology and hospital recruited from. It is important to note that these studies were not specific to liver mortality but included adverse liver outcomes such as decompensation and HCC in their analysis.

Whilst small study numbers limits any conclusions taken from the Newcastle ELF analysis, clear trends can be seen in associated mortality risk with "high risk" ELF scores. Further research with NAFLD specific cohorts, over a long follow-up duration are required to better understand any role the ELF panel may play in identifying those patients at risk of all-cause mortality. Given the small numbers of deaths in the Newcastle ELF cohort further study into liver-specific cause of death was not possible, but again this would be an area of great interest for future research. It is likely alternative cut-offs would need to be established for the prediction of liver outcomes and mortality, and larger studies would be needed to validate this further.

Results from a large international NAFLD drug trial identified that higher baseline ELF results were associated with disease progression (HR 2.58, 95% CI 1.96 – 3.38, p<0.001) and a greater change in repeat ELF result was also significantly associated.[172] This study which had a mean follow-up of 30.9 months also identified that a higher baseline ELF score was associated with an increased risk of a liver related event (HR 2.11, 95% CI 1.53 – 2.90, p<0.001). This is one of the main NAFLD specific studies to explore the use of the ELF panel to predict outcomes, but it is important to note that when predicting liver related outcomes, they suggest the use of alternative cut-offs to that currently used in the U.K. from the NICE guidelines (≥11.27, sensitivity 51%, specificity 72%). The short duration of this study meant that few participants had reached liver outcomes of interest such as decompensation, HCC or mortality and follow-up over a longer period is required to further explore these important outcomes.

This Newcastle ELF study does have some limitations. As briefly discussed above the study cohort was small and as a result the statistical power to demonstrate significance of the ELF as a predictor of outcomes is limited. The study is also retrospective, and the cohort are derived from a tertiary centre which will have created a selection bias towards more advanced disease and therefore potentially increase the rates of clinical outcomes such as

the development of HCC or death. Due to the duration of follow-up, there were also a number of missing data points for this study, which may result in some cases of disease progression, diagnosis of co-morbidities and clinical outcomes such as development of HCC having been missed. This long length of follow-up however is also a strength of this cohort to assess for clinical outcomes of interest in what is known to be a slowly progressive condition. The strengths and weaknesses of the entire Newcastle NAFLD study will be fully discussed in the following chapter.

Chapter 8: General Discussion

8.1. Summary of study findings

Since it was first described as an aetiology separate from alcohol related fatty liver disease in 1980,[202] non-alcoholic fatty liver disease has become a leading cause of chronic liver disease worldwide.[80] NAFLD has generally been shown to be a slowly progressive condition, with only 15-30% progressing to advanced fibrosis or cirrhosis.[96] Despite this, due to the high prevalence of the condition in the community, there has been an increasing incidence of complications, including decompensated liver disease and hepatocellular carcinoma, and NAFLD is set to become the leading cause of liver transplantation in the next decade.[83]

The purpose of this study was to explore the long-term outcomes of a cohort of over 600 individuals with NAFLD from a single U.K. centre for up to 35 years to gain a better understanding of the frequency of significant clinical events and look at factors predicting the occurrence of these events.

Chapter 3 described the baseline characteristics of the Newcastle NAFLD cohort, which comprised of 605 patients, 95% of whom underwent liver biopsy at baseline. A wide range of disease spectrum was found, with all stages of fibrosis (F0-4) seen and 62% meeting the criteria for NASH. A fifth (19.2%) of the cohort had cirrhosis and the FIB-4 and NFS scores were both found to perform well in identifying advanced fibrosis and cirrhosis. There were high incidences of co-morbidities such as hypertension, the metabolic syndrome and IHD, and in keeping with other studies, a diagnosis of T2DM was associated with an increased risk of cirrhosis, even after adjusting for BMI, age, and sex.

The follow-up events for the cohort were described in the following chapter. There were 112 deaths during the study, with a mean time to death of 10 years, and 28.5% of these were due a liver related death. Several factors were identified as independent risks for death including the presence of IHD and “high-risk” FIB-4 scores.

Chapter 5 then further expanded on the group of 42 individuals who met the criteria of “liver mortality”, defined as a liver related death or transplantation, over the course of the study. This chapter explored the characteristics of these patients in greater detail than has been reported in the general literature to date and identified that along with fibrosis stage,

the presence of T2DM and IHD were associated with an increased risk of liver death. In addition, “high risk” FIB-4 and NFS scores could also prognosticate for liver mortality.

Liver outcomes such as fibrosis progression, diagnosis of cirrhosis and development of HCC were discussed in the following chapter. High steatosis score on baseline histology was a predictor of histological disease progression, along with T2DM, while clinical progression to cirrhosis was once again associated with “high-risk” FIB-4 scores.

The final results chapter examined a smaller sub-cohort of individuals who had been involved in the 2008 study by Guha and colleagues in the validation of the ELF score. These results were re-explored and compared to the FIB-4 and NFS scores, which found all these scores had good accuracy in identifying advanced fibrosis (F3-4). Each score was also examined in prognostic ability for all-cause mortality and the “high risk” category for each was associated with an increased risk of death.

8.2. Strengths and weaknesses of studies

The Newcastle NAFLD study is the largest, single centre, U.K. based NAFLD cohort study with one of the longest follow-up periods reported in the literature. One of the biggest strengths of the study is the well characterised cohort with over 95% of individuals biopsy confirmed at the time of baseline, reviewed by only two histopathologists who worked closely together. At the time of the baseline visit, patients with a wide range of histological severity and disease burden were included, which allowed follow-up of the outcomes of the full spectrum of NAFLD, including those with NAFL at baseline. The study collected data relating to the individual’s full health and thus was able to provide a detailed narrative of the entire Newcastle NAFLD cohort, including a comprehensive and accurate description of the causes of death. This study reflects a “real-world” clinical NAFLD cohort in the U.K. with a very long follow-up period, up to 35 years, and the results are therefore likely to be broadly applicable to real world clinical practice.

This study does however have some limitations, which have been briefly discussed in the preceding chapters. Firstly, the study design is retrospective, and recruitment was undertaken in a tertiary level hepatology department. This is likely to have biased the cohort towards more advanced disease, which in turn may have increased the occurrence of events of clinical interest, such as a diagnosis of cirrhosis and its complications. Secondly, the

missing data points may have inadvertently biased results as the largest proportions of unavailable data were historical variables that were not collected at the initiation of the study, but later added to the NAFLD registry. Some baseline data from patients recruited early in the study were therefore not available. Moreover, a significant proportion of the cohort were lost to follow up meaning that as rich a clinical dataset was not available for these individuals. Thirdly, the small numbers of occurrences of some outcomes of interest, such as the number of individuals included in the ELF cohort or those who development of HCC during the study, will have limited the statistical power of the analysis of these outcomes. Finally, the study cohort is representative of a tertiary centre from the North-East of England which lacks diversity, reflective of the local population, and the individuals who remained under routine clinical care were regularly given lifestyle advice which may bias outcomes compared to a general population.

8.3. Future implications

Throughout the outcomes of this long-term NAFLD study, non-invasive scores including the FIB-4 and NFS, have been found to perform well in identifying advanced disease as they are designed to do, but both also have been shown to identify those at higher risk of disease progression and adverse outcomes including all cause death. These findings add to the growing body of evidence indicating that these scores could have prognostic uses. However, large, prospective studies are required to further understand their potential. The use of sequential scores at follow-up visits would also warrant prospective investigation and could provide a useful tool in the clinical management of risk of those with NAFLD in the longer-term. Within the Newcastle NAFLD cohort, further exploration of the FIB-4 and NFS results recorded at annual reviews in between the clinical events described in this thesis (baseline and final event) should be undertaken as a priority. In particular their ability to predict clinical events and histological change should be examined.

Cardiovascular disease is well known to have a complicated relationship with NAFLD, with more recent studies suggesting it may contribute to disease progression. Thus far there is a paucity of evidence as to the influence CVD may have on outcomes in NAFLD including development of HCC or death. The Newcastle NAFLD cohort has found the presence of IHD is associated with an increased risk of such adverse outcomes and further prospective studies could further explore the effect of co-existent heart disease, and any value it may have in a

clinically derived prognostic model. Further examination of other, less reported, major adverse cardiovascular events such as peripheral vascular disease and stroke should also be undertaken given the rich data collected by the Newcastle NAFLD cohort.

The relationship between T2DM and NAFLD should also be further explored in the Newcastle NAFLD cohort. Examining any relationship between HbA1c control and clinical events would add to the clinical understanding of how diabetes management should be approached in NAFLD patients and how this may differ from those who do not have NAFLD.

As discussed in chapter 7, the use of the ELF panel has become controversial in recent years with the changes in algorithms and laboratory processing. The Newcastle NAFLD ELF cohort has shown good correlation between the score and disease stage, but also its potential use as a prognostic marker. Repeat ELF assessment within the Newcastle NAFLD cohort and further prospective studies to explore the use of the score in prognosticating NAFLD are required.

Finally, to fully understand the U.K. population burden of NAFLD and the long-term outcomes a large, prospective, general population study is warranted.

Appendices

Appendix A: Patient cover letter

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The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

The Freeman Hospital
High Heaton
Newcastle upon Tyne
NE7 7DN

Tel: 0191 233 6161
Fax: 0191 2137297

Dear

The Hepatology Department at the Newcastle Upon Tyne Hospitals NHS Trust and Newcastle University are trying to understand the long-term implications of being diagnosed with "Non-Alcoholic Fatty Liver Disease" (NAFLD), a condition that you were diagnosed with following a liver biopsy in the Freeman Hospital some years ago.

The aim of the study is to gain a better understanding of the long-term outcomes of NAFLD by reviewing the medical records of patients who have undergone a liver biopsy at the Newcastle Hospitals over the past 30 years in order to find out what other medical problems people may have experienced over the following years.

I have enclosed a Patient Information Sheet which contains more details regarding the study with this letter and would be very grateful if you could read this.

The NHS Health Research Authority have granted their approval for the Hepatology Research Team at the Newcastle Hospitals Trust to access existing medical records of patients diagnosed with NAFLD in order to collect relevant data which will help better our understanding of this condition. As a patient previously under the care of the Newcastle Trust we are writing to inform you of our intention to access your medical records as part of this study. This research has been fully reviewed and approved by an NHS Health Research Authority Research Ethics Committee and the Confidentiality Advisory Group (CAG).

If you have any questions regarding this study, or decide you would not like your information to be included in the study, please get in touch with my colleague Dr Jennifer Gallacher either by phone; 0191 213 9938 or by email; Jennifer.gallacher@nuth.nhs.uk .

Yours Sincerely,

Professor Quentin M. Anstee PhD, FRCP

Professor of Experimental Hepatology & Honorary Consultant Hepatologist

Appendix B: Original Patient Information Sheet

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Long-Term Outcomes of Non-Alcoholic Fatty Liver Disease

Patient Information Sheet

Introduction

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others, if you wish. If anything is unclear, or if you would like further information, please feel free to ask the person who gave you this leaflet or speak to a member of the research team directly on 0191 213 9938. Take time to decide whether you wish to take part.

NAFLD is a liver problem that is caused by fat collecting in the liver and can lead to liver inflammation (this is called "steatohepatitis"), liver scarring, cirrhosis and liver cancer. It affects almost a quarter of the population of the United Kingdom but only a small percentage of those who have NAFLD will be badly affected by the condition. As its name suggests, it is not caused by drinking too much alcohol. Accurate diagnosis of fatty liver disease is difficult, often requiring a liver biopsy and currently there are very few medicines or treatments that are proven to work.

NAFLD often occurs in people who have diabetes, high cholesterol, and high blood pressure or are overweight. We hope to gain a better understanding about why NAFLD affects some people more severely than others. To do this, we need to know more about the subtle person-to-person differences that make each of us unique, and what happens to people that have been diagnosed with NAFLD over the long-term. As part of this study, we will store information about you, such as your age, the results of your blood tests and scans, your medical conditions and the treatments that you have received. We will combine your information with details from hundreds of other people and look across the whole group. We expect that these subtle variations will explain the differences in how much fatty liver impacts on people's health.

This study is led by doctors at the Freeman Hospital and Newcastle University, and is part of an educational project for Dr Jenny Gallacher who will use this research as part of her M.D. thesis in liver disease.

It has been funded by a medical research grant called LITMUS from the European Commission under the Innovative Medicines Initiative (IMI2) scheme. Members of the research team include specialist doctors and researchers at universities across Europe as well as companies developing better tests or treatments for liver disease. You will not personally receive any financial benefit from taking part in the research.

Why have I been chosen?

You are being asked to participate because you have been diagnosed with non-alcoholic fatty liver disease (NAFLD, "fatty liver") on a liver biopsy previously done at the Newcastle Hospitals.

Do I have to take part?

No. It is entirely up to you to decide whether or not to take part and the decision not to take part will not affect your medical care in any way.

In order to make it as simple as possible for participants we have designed the study using an opt-out consent process.

If you do **NOT** wish for your data to be included please complete the opt-out form enclosed and return it using the self-addressed envelope.

If we have not received this form from you in 6 weeks we will assume you are happy to take part and will start data collection. However you are free to withdraw at any point after this time.

What will happen to me if I take part?

Participating in the study does not involve any further hospital appointments or tests being done. All information collected is already documented in medical records.

Following this letter, you will receive no further contact from the study.

We will record some information about your health in a secure computer database (the European NAFLD Registry). We will collect information like your age, date of birth, gender, test results (for example, blood tests, biopsies or scans) and information on any other illnesses you may have or the treatment that you receive. Where possible we will access electronic data, such as your blood test results and your treatment details, which are held on General Practice and Hospital computer systems. The information will be collected in what is called "linked-anonymised" or "codified" form, which means that we will not record identifiable information like your name or address but will use a unique code number instead. All coded information on you will be kept in accordance with the relevant national and international regulations.

No information or results linked to your identity will be released to the public or published.

No personal data will leave the hospital site.

What are the Benefits of taking part?

It is unlikely that you will personally benefit from participation in this study. We hope, however, that the outcome of the research could help improve the care of other patients in the future.

What are the Disadvantages of taking part?

There is no need for any additional hospital visits or other tests due to taking part in this study so taking part should not cause any inconvenience. Participating in the study will have no effect on your future care nor will it impact in any other personal arrangements such as insurance.

Who has reviewed the study?

The North East- Newcastle & North Tyneside 1 Research Ethics Committee, which has responsibility for reviewing medical research studies, has approved this study. In addition, the study has been extensively reviewed by medical experts and an ethics panel appointed by the European Commission under the Innovative Medicines Initiative (IMI2) funding scheme. An independent "Ethics Guardian" has been appointed to oversee the ethical conduct of the entire project.

Who is organising and funding the research?

Professor Quentin Anstee is the lead investigator responsible for overseeing the conduct of the European NAFLD Registry project. The Newcastle upon Tyne Hospitals NHS Foundation Trust (NUTH) is the sponsor for this study. The research is being undertaken by an international research consortium called LITMUS, which is made up of leading medical specialists from universities and hospitals across Europe, working in collaboration with companies that are developing diagnostic tests or new treatments for fatty liver disease. The LITMUS European NAFLD Registry project is funded by the European Commission under the Innovative Medicines Initiative (IMI2) funding scheme and is also coordinated by Professor Quentin Anstee, Newcastle University, UK.

We will be using information from your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. The Newcastle Hospitals Trust will keep identifiable information about you for 15 years.

How will my privacy be protected?

Information collected about you at the research site. If you decide to join the study, the Newcastle upon Tyne Hospitals Foundation Trust (NUTH) will collect information about you for research from your past medical records and test results. This information will include details such as your name, sex at birth, date of birth or age and ethnicity. The study doctor and his/her team will keep this information confidential, and will not provide any identifying information about you to NUTH, using it for the following purposes (and retaining it for the time periods required by law):

- Conducting this study and complying with regulatory duties: such as ensuring compliance with the legal requirements of the clinical trial at the site; answering enquiries of regulatory authorities and/or ethical committees or competent authorities (domestic or foreign) to verify the proper conduct of the study.
- Undertaking end of study data collection and a life status check with public sources if it is not possible to reach you.

Information sent outside the research site. Any information about you that is sent outside the research site (for example, study documents and data, including results of tests) will be assigned a code by the study team. This code replaces your name when sending information to the sponsor or other recipients. This code will be collected and stored in the database for the study with the other information about you, such as clinical information obtained during the conduct of the study (for example, blood pressure, weight, test results). The study doctor and the study team will maintain the key to the code (the key allows you to be identified if needed, for example, for safety reasons).

Your coded personal information will be used by LITMUS (alone or in collaboration with public or private partnerships in the area of scientific research, such as other members of LITMUS, including academic entities and diagnostic and pharmaceutical companies) for the following purposes:

- Learning more about key stages of the progression of liver disease.
- Publishing scientific works and using them for educational purposes
- Conducting further scientific research activities

When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research.

This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information by contacting Dr Jenny Gallacher using the below details.

Contact for Further Information

Local Principal Investigator: Dr Jenny Gallacher
Freeman Hospital
Jennifer.gallacher@nuth.nhs.uk
0191 213 9938

Other Local Contacts: Hepatology Research at Newcastle-upon Tyne Hospitals
0191 223 8429

Chief Investigator: Prof Quentin M. Anstee
Newcastle University

quentin.anstee@newcastle.ac.uk

For independent information about participating in research studies or to get advice please contact LIVErNORTH (http://www.livernorth.org.uk, Telephone 0191 3702961), the British Liver Trust (http://www.britishlivertrust.org.uk/) or your local Patient Advice and Liaison service (Freephone 0800 032 0202).

Thank you very much for taking the time to read and consider your participation in this study.

Appendix C: Patient Information Sheet post CAG feedback

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Long-Term Outcomes of Non-Alcoholic Fatty Liver Disease

Patient Information Sheet

Your medical information has been selected to be included in this study because you have previously been diagnosed with non-alcoholic fatty liver disease (NAFLD, "fatty liver") on a liver biopsy done at the Newcastle Hospitals some time ago.

What is NAFLD?

NAFLD is a liver problem that is caused by fat collecting in the liver and can lead to liver inflammation (this is called "steatohepatitis"), liver scarring, cirrhosis and liver cancer. It affects almost a quarter of the population of the United Kingdom but only a small percentage of those who have NAFLD will be badly affected by the condition. As its name suggests, it is not caused by drinking too much alcohol. Accurate diagnosis of fatty liver disease is difficult, often requiring a liver biopsy and currently there are very few medicines or treatments that are proven to work.

NAFLD often occurs in people who have diabetes, high cholesterol, and high blood pressure or are overweight. We hope to gain a better understanding about why NAFLD affects some people more severely than others. To do this, we need to know more about the subtle person-to-person differences that make each of us unique, and what happens to people that have been diagnosed with NAFLD over the long-term. As part of this study, we will store information about you, such as your age, the results of your blood tests and scans, your medical conditions and the treatments that you have received. We will combine your information with details from hundreds of other people and look across the whole group. We expect that these subtle variations will explain the differences in how much fatty liver impacts on people's health.

This study is led by doctors at the Freeman Hospital and Newcastle University, and is part of an educational project for Dr Jenny Gallacher who will use this research as part of her M.D. thesis in liver disease.

It has been funded by a medical research grant called LITMUS from the European Commission under the Innovative Medicines Initiative (IMI2) scheme. Members of the research team include specialist doctors and researchers at universities across Europe as well as companies developing better tests or treatments for liver disease.

Does my data have to be included?

No. If after reading this information sheet you have any questions about your data being included please contact Dr Jennifer Gallacher (details below) who can answer any queries you may have or remove you from the study if you decide not to participate.

What will happen to me if my data is included?

Participating in the study does not involve any further hospital appointments or tests being done.
All the information that is needed for the study is already documented in your medical records and with NHS Digital. Following this letter, you will receive no further contact from the study.

We will record some information about your health in a secure computer database (the European NAFLD Registry). We will collect information like your age, date of birth, gender, test results (for example blood tests, biopsies or scans) and information on any other illnesses you may have or the treatment that you receive. Where possible we will access electronic data, such as your blood test results and your treatment details, which are held by NHS Digital or on General Practice and Hospital computer systems. The information will be collected in what is called "linked-anonymised" or "codified" form, which means that we will not record identifiable information like your name or address but will use a unique code number instead. All coded information on you will be kept in accordance with the relevant national and international regulations.

No information or results linked to your identity will be released to the public or published.

What are the Benefits?

It is unlikely that you will personally benefit from participation in this study. We hope, however, that the outcome of the research could help improve the care of other patients in the future.

What are the Disadvantages of my data being included?

There is no need for any additional hospital visits or other tests due to taking part in this study so taking part should not cause any inconvenience. Participating in the study will have no effect on your future care nor will it impact in any other personal arrangements such as insurance.

Who has reviewed the study?

The North East- Newcastle & North Tyneside 1 Research Ethics Committee, which has responsibility for reviewing medical research studies, has approved this study. We have also sought approval from the NHS Confidentiality Advisory Group to gain permission to access this information. In addition, the study has been extensively reviewed by medical experts and an ethics panel appointed by the European Commission under the Innovative Medicines Initiative (IMI2) funding scheme. An independent "Ethics Guardian" has been appointed to oversee the ethical conduct of the entire project.

Who is organising and funding the research?

Professor Quentin Anstee at Newcastle University is the lead investigator responsible for overseeing the conduct of the European NAFLD Registry project. The Newcastle upon Tyne Hospitals NHS Foundation Trust (NUTH) is the sponsor for this study. The research is being undertaken by an international research consortium called LITMUS, which is made up of leading medical specialists from universities and hospitals across Europe, working in collaboration with companies that are developing diagnostic tests or new treatments for fatty liver disease. The LITMUS European NAFLD Registry project is funded by the European Commission under the Innovative Medicines Initiative

(IMI2) funding scheme and is also coordinated by Professor Quentin Anstee, Newcastle University, UK.

We will be using information from your medical records in order to undertake this study. Newcastle University will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. The Newcastle Hospitals Trust will keep identifiable information about you for 15 years.

How will my privacy be protected?

Information collected about you at the research site. The Newcastle upon Tyne Hospitals Foundation Trust (NUTH) will collect information about you for research from your past medical records and test results. This information will include details such as your name, sex at birth, date of birth or age and ethnicity. The study doctor and his/her team will keep this information confidential, and will not provide any identifying information about you to NUTH, using it for the following purposes (and retaining it for the time periods required by law):

- Conducting this study and complying with regulatory duties: such as ensuring compliance with the legal requirements of the clinical trial at the site; answering enquiries of regulatory authorities and/or ethical committees or competent authorities (domestic or foreign) to verify the proper conduct of the study.
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- Learning more about key stages of the progression of liver disease.
- Publishing scientific works and using them for educational purposes
- Conducting further scientific research activities

The information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research.

This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

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You can find out more about how we use your information by contacting Dr Jenny Gallacher using the below details.

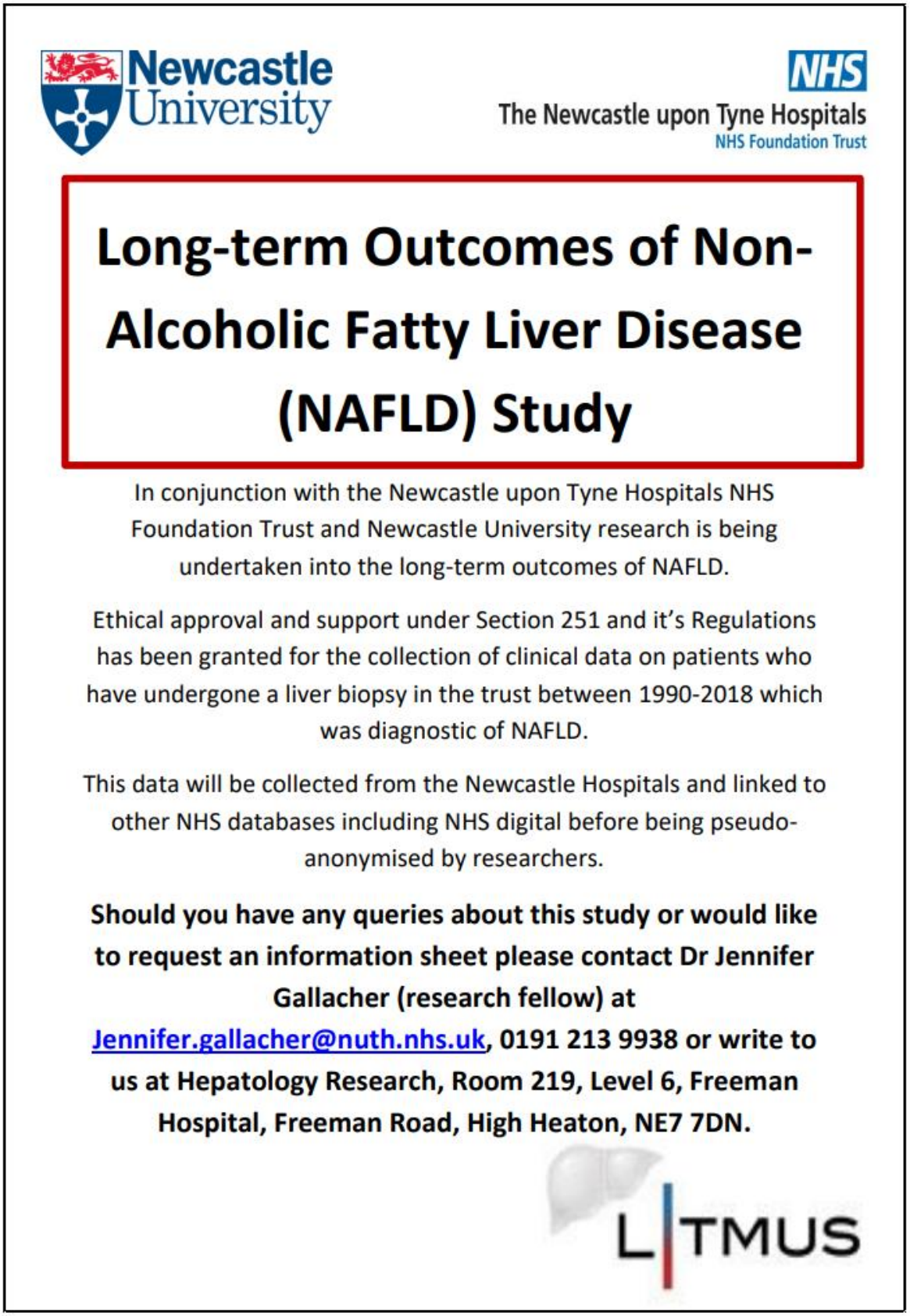
Contact for Further Information

Local Principal Investigator: Dr Jenny Gallacher
Freeman Hospital
Jennifer.gallacher@nuth.nhs.uk
0191 213 9938

Other Local Contacts: Hepatology Research at Newcastle-upon Tyne Hospitals
0191 223 8429

Chief Investigator: Prof Quentin M. Anstee
Newcastle University

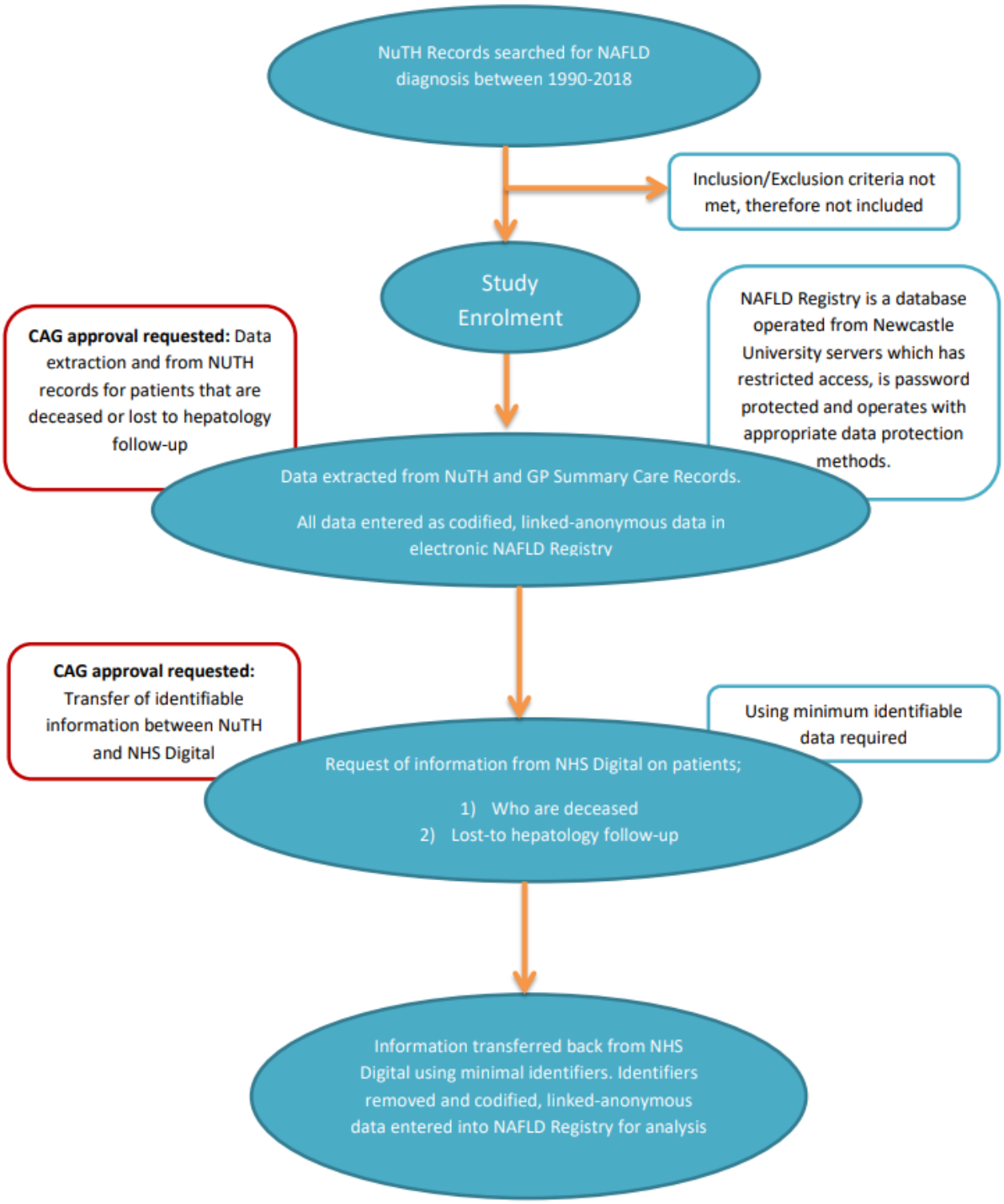
Appendix D: Study Poster



Appendix E: CAG Dataflow Diagram

NAFLD Long-term Outcomes Study Dataflow Diagram.

CAG Application 19/CAG0095



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