Non-Alcoholic Fatty Liver Disease: Disease burden and development of novel fibrosis diagnostic and prognostic signatures

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ABSTRACT

Background This thesis investigates novel diagnostic and prognostic disease biomarkers in NAFLD and explores both the quality of life (QoL) and economic burden associated with NAFLD.

Methods To estimate HRQL burden, 147 patients completed validated QoL assessments within 6 months of diagnostic liver biopsy. NAFLD out-patient service utilisation was evaluated and micro-costed over a 12-month period. The clinical utility of serum collagen neo-epitope biomarkers to identify advanced fibrosis was established. DNA methylation was evaluated in circulating cell free DNA as a diagnostic biomarker in NAFLD using pyrosequencing and evaluated by whole genome bisulfide sequencing (WGBS) from paired liver biopsy tissue to characterise NAFLD prognostic signatures.

Results ***HRQL Burden:*** Grade of lobular inflammation influenced CLDQ scores and FIS scores. One way ANCOVA analyses showed that CLDQ scores were influenced by fibrosis stage (F=1.910, p=0.014, effect size 0.814) ***Economic Burden:*** Multivariate regression analysis established the main cost drivers to be the number of clinic appointments (p=0.042) and the presence of advanced disease (p=0.001). ***Collagen Neo-epitope biomarkers*** the novel "FIBC3" diagnostic panel including PROC3 exhibited improved accuracy and outperformed other fibrosis indices for the detection of advanced fibrosis ***DNA methylation fibrosis biomarkers*** PPARγ CpG methylation displayed uniform hypermethylation at each CpG site between the liver fibrosis cohorts relative to uniform hypomethylation irrespective of liver disease aetiology ***DNA methylation prognostic signature;*** > 657 novel methylation signatures to distinguish low and high risk disease were identified.

Conclusion Multiple factors negatively impact on reported HRQL, notably fatigue and lobular inflammation. The direct medical costs associated with NAFLD are substantial and increase with the presence of advanced disease. The 'FIBC3' panel is an accurate tool with a single threshold value that maintains both sensitivity and specificity for the identification of advanced fibrosis (F≥3). The first methylome map of low versus high risk disease in NAFLD suggest that high and low risk NAFLD while interrelated, may be biologically distinct from disease onset. Extending this towards clinical utility, uniform hypermethylation at the PPARγ gene promoter confirms this as a potential methylation signature for fibrosis progression in chronic liver disease.

TABLE OF CONTENTS

INDEX OF FIGURES

TABLE OF TABLES

ABBREVIATIONS

5mC	5-Methylcytosine
A2MP1	Alpha-2-Macroglobulin Pseudogene 1
AAMP	Angio associated migratory cell protein(AAMP)
AAR	AST/ALT Ratio
AASLD	American Association for the study of liver disease
ACR	American College of Rheumatology
ADAMS2	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
ADIPOR2	Adiponectin Receptor 2
ADRA2A	Adrenoceptor Alpha 2A
AI	Artificial Intelligence
AIH	Auto-immune hepatitis
ALD	Alcoholic Liver Disease
ALT	Alanine aminotransferase
AMBP	Alpha-1-Microglobulin/Bikunin Precursor
AMP	Association of Molecular Pathology
ANCOVA	Analysis of covariance
APRI	AST to platelet ratio index
ARFI	Acoustic Radiation force impulse imaging
ARHGEF37	Rho Guanine Nucleotide Exchange Factor 37
ASP	Average Sleep Propensity
AST	Aspartate aminotransferase
ATG16L2	autophagy related 16 like 2(ATG16L2)
AUROC	Area under the receivor operating curve
BAMBI	BMP And Activin Membrane-Bound Inhibitor Homolog
BASL	British Association for the study of liver disease
BHLHE41	Basic helix-loop-helix family member e41
BIPED	Burden of disease, Investigative, Prognostic, Efficacy of intervention, Diagnostic
BIV2	Beck's Inventory Version 2
BM	Basement Membrane
BMI	Body Mass Index
BMP4	Bone Morphogenetic Protein 4
Bps	Base pairs
BRIC	Benign Recurrent Intrahepatic cholestasis
BS	Bisulfite sequencing
BSG	Basigin
C1QTNF8,	Complement C1q tumour necrosis factor-related protein 8
C3M	Collagen Degradation marker 3
C3orf38	Chromosome 3 open reading frame 38
C4M	Collagen Degradation marker 4
CAP	College of American Pathologists

ccfDNA	Cell free circulating DNA
CD300A	Cluster of Differentiation 300A
CEN	Technical Specifications (CEN/TS)
CFAP298	Cilia and Flagella Associated Protein 298
CGGBP1	CGG triplet repeat-binding protein 1;
CGIs	CpG islands
CHD7	Chromodomain helicase DNA binding protein 7
CI	Confidence interval
CK-18	Cytokeratin 18
CKD	Chronic Kidney Disease
CLD	Chronic liver disease
CLDQ	chronic liver disease questionnaire
CLIP2	CAP-Gly Domain Containing Linker Protein 2
CNN	Convolutional neural networks
COI	Cost of illness
CpG	5'—C—phosphate—G—3'
CRN	Clinical Research Network
CST1	Cystatin SN
CT	Computerised Tomography
CTX1	C-terminal telopeptide
D2	Dopamine receptors
DILI	Drug induced liver injury
DLCO	Diffusing capacity of the lungs for carbon monoxide
DLS	Deep Learning system
DML	Differentially methylated loci
DMR	Differentially methylated region
DMXL1	Dmx like 1(DMXL1)
DNA	Deoxyribonucleic acid
DOK1	Docking protein 1
DPP9	Dipeptidyl Peptidase 9
DT	Dina Tineokas
DUSP3	Dual specificity protein phosphatase 3
ECM	Extracellular matrix
EDEM2	ER Degradation Enhancing Alpha-Mannosidase Like Protein 2
EDTA	Ethylenediaminetetraacetic Acid
EEF1AKMT1	EEF1A Lysine Methyltransferase 1
EIF3J	Eukaryotic Translation Initiation Factor 3 Subunit J
ELF	Elevated Fibrosis Panel
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicine Agency
EOGT	EGF Domain Specific O-Linked N-Acetylglucosamine Transferase
EPDR1	Ependymin Related 1

EPN1	Epsin 1
EPoS	Elucidating pathways in Steatohepatitis
EPR	Electronic patient records
EPS15	Epidermal Growth Factor Receptor Pathway Substrate 15
ESRRA	Estrogen related receptor alpha(ESRRA)
ESRRG	Estrogen Related Receptor Gamma
ESRRG	Estrogen related receptor gamma(ESRRG)
ESS	Epworth Sleepiness Scale
EULAR	European League against Rheumatology
EXT2	Exostosin Glycosyltransferase 2
FADS2	Fatty Acid Desaturase 2
FAIM	Fas apoptotic inhibitory molecule 1.
FBLL1	Fibrillarin Like 1
FDA	Food and Drug Agency
FFA	Free fatty acids
FFPE	Fixed formalin paraffin embedded
FIB-4	Fibrosis 4 Index
FIS	Fatigue Impact Scale
FP	Fast Progressor
FPA	Fibrinopeptide A
FPGS	Folylpolyglutamate synthase
FSR	Fractional synthesis rate
FXR	Farnesoid X receptor
GGT	Gamma-glutamyltransferase
GI	Gastrointestinal
GPBP1L1	GC-Rich Promoter Binding Protein 1 Like 1
GPR25	Orphan G protein-coupled receptor 25
GSEA	Gene Set Enrichment Analysis
HA	Hyaluronic Acid
HALY	Health-Adjusted Life Years
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCK	Tyrosine-protein kinase
HCV	Hepatitis C Virus
HGFAC	Hepatocyte growth factor activator
HM13	Minor histocompatibility antigen H13
HRQL	Health related Quality of Life
HSC	Hepatic stellate cells
HVPG	Hepatic venous pressure gradient
IBD	Inflammatory Bowl Disease
ICAN	Institute of Cardiometabolism and Nutrition Paris
ICM	Institute of cellular medicine

ICTP	Type I collagen degradation product
IMI	Innovative Medicine Initiative
IR	Insulin Resistance
ISO	international organization of standardizations
ISOQOL	International Society for Quality of Life Research
ISX	Intestine Specific Homeobox
IU	International Units
KCTD3	Potassium channel tetramerization domain containing 3(KCTD3)
KDSR	3-Ketodihydrosphingosine Reductase
LAIR1	Leukocyte Associated Immunoglobulin Like Receptor 1
LCM	Laser Capture Microdissection
LFT	Liver function tests
LI	Lobular inflammation
LILRB1	Leukocyte immunoglobulin-like receptor subfamily B member 1
LINC00987	Long Intergenic Non-Protein Coding RNA 987
LITMUS	Liver Investigation: Testing Marker Utility in Steatohepatitis
LLGL2	LLGL2, scribble cell polarity complex component(LLGL2)
LMNTD2	Lamin B2
LMR	Low methylation regulatory elements
LOXL2	Lysyl oxidase-like-2
LST1	Leukocyte Specific Transcript 1
LTB4R2	Leukotriene B4 Receptor 2
MAO	Mono-amine oxidase
MBDs	Methyl-CpG binding domains
MCTS2P	Malignant T cell amplified sequence 2
MeCp2	Methyl-CpG Binding Protein 2
MEGF6	Multiple EGF Like Domains 6
MetS	Metabolic Syndrome
MGLL	Monoglyceride Lipase
MRE	MRI Elastography
MRI	Magnetic Resonance Imaging
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	NASH activity score
NASH	Non-alcoholic steatohepatitis
NCF2	Neutrophil Cytosolic Factor 2
NEMO	Nuclear factor κB essential modulator
NFS	NAFLD Fibrosis Score
NGS	Next Generation sequencing
NHS	National Health Service
NIH	National Institute of Health
NPV	Negative Predictive Value

NR2F1-AS1	NR2F1 antisense RNA 1
NR2F6	Nuclear receptor subfamily 2 group F member 6(NR2F6)
OA	Osteoarthritis
OLT	Orthotopic Liver Transplant
OR	Odds ratio
OSA	Obstructive Sleep apnoea
PAF	Population-attributable fraction
PBC	Primary Biliary Cirrhosis
PCOS	Polycystic Ovarian Syndrome
PDGFα	Platelet Derived Growth factor alpha
PEN	Polyethylene naphthalate
PGF	Platelet Growth factor
PHC	Personalised Healthcare
PIEZO 2	Piezo Type Mechanosensitive Ion Channel Component 2
PIIINP	Pro-peptide of type III collagen
PMD	Partially methylated domains
PNPLA3	Patatin-like phospholipase domain containing 3
PPARα	Peroxisome Proliferator Activated Receptors
PPARγ	Peroxisome Proliferator Activated Receptors
PPM1L	Protein Phosphatase, Mg2+/Mn2+ Dependent 1L
PPV	Positive Predictive Value
PRDM10	Prosite-Prorule /SET Domain 10
PRO	Patient Reported Outcome
PROC3	Pro-collagen 3
PROC4	Pro-collagen 4
PROC6	Pro-collagen 6
PSC	Primary sclerosing cholangitis
PTX3	Pentraxin 3
QALY	Quality-Adjusted Life Years
Qc	Quality control
QoL	Quality of life
RAI1	Retinoic Acid Induced 1
RASSF1	Ras Association Domain Family Member 1
RB1	Retinoblastoma-associated protein
RCE1	Ras Converting CAAX Endopeptidase 1
RCN 1	Reticulocalbin 1
RNA5SP155	RNA, 5S Ribosomal Pseudogene 155
ROI	Region of interest
RORC	RAR related orphan receptor C(RORC)
ROS	Reactive Oxygen Species
Rs	Spearmann rank correlation
RXR	retinoid X receptor

SACS	Sacsin
SD	Standard deviation
SDH	succinate dehydrogenase
SDHAF4	Succinate dehydrogenase assembly factor 4
SDK1	Sidekick Cell Adhesion Molecule 1
SEER	Surveillance, Epidemiology, and End Results database
SF-36	Short Form Health Survey
SH	Steatohepatitis
SHIP	Study of Health in Pomerania
SIBO	Small intestinal bacterial overgrowth
SIM1	Single-Minded Homolog 1
SISAQOL	Setting International Standards in Analysing Patient Reported Outcomes and Q of Life Endpoints Data
SIX5	SIX Homeobox 5
SNP	Single nucleotide polymorphism
SPIDIA	Standardisation and improvement of pre-analytical procedures in vitro diagnos
SR	Stable/Regressors
SRSF10	Serine and Arginine Rich Splicing Factor 10
SSc	Systemic Sclerosis
SSI	Supersonic shear wave elastography
STAG3L3	Stromal Antigen 3-Like 2
T2DM	Type 2 Diabetes Mellitus
TACC1	Transforming Acidic Coiled-Coil Containing Protein 1
TBL3	Transducin beta like 3(TBL3)
TCF7L1	Transcription Factor 7 Like 1
TET1	Tet Methylcytosine Dioxygenase 1
TGF	Transforming Growth factor
TM6SF2	Transmembrane 6 Superfamily Member 2
TMF1	ATA Element Modulatory Factor 1
TONSL	Tonsoku Like, DNA Repair Protein
TWSG1	Twisted Gastrulation BMP Signalling Modulator 1
TSS	Transcriptional Start Site
U2AF2	U2 Small Nuclear RNA Auxiliary Factor 2
UK	United Kingdom
UM	University Hospital Mainz
UNEW	Newcastle University
UNITO	University of Torino
UNOS	The United Network Organ Sharing
UP	University of Palermo
USA	United States of America
USP	University of Sao Paulo School of Medicine

USP53	Ubiquitin carboxyl-terminal hydrolase 53
USS	Ultrasound
VCTE	Vibration-controlled elastography
Vwf	Van Willebrand factor
WDR1	WD repeat domain 1(WDR1)
WDR12	WD repeat domain 12(WDR12)
WDR78	WD repeat domain 78(WDR78)
WGBS	Whole genome bisulfide sequencing
YI	Youden Index
ZNF654	Zinc Finger Protein 654
ZNF670	Zinc Finger Protein 670

CO-AUTHOR CONTRIBUTIONS

CHAPTER 2:

Exploring emotional, mental, physical and social functioning in NAFLD: Data from the European NAFLD Registry; an analysis of Patient reported outcomes (PROs) in NAFLD

Study conception and design	Marie Boyle (MB), Jorn Schattenberg (JS), Professor Quentin Anstee (QMA)
Acquisition of data	Elsbeth Henderson (EH) distributed Patient Reported Outcomes (PROs) in hepatology outpatients, Freeman hospital, Newcastle. Supplementary CLDQ data was obtained from EPoS consortium members in the University Centre of the Johannes Gutenberg-University Mainz, Germany and the University Hospital of Seville for comparative purposes
Analysis and interpretation of data	MB
Drafting of chapter	MB
Critical revision	QMA

CHAPTER 3:

Cost of illness study associated with the prevalence, severity and patterns of clinical practice in outpatient visits for NAFLD – the United Kingdom CONSTANS STUDY

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Acquisition of data	MB
Analysis and interpretation of data	MB
Drafting of chapter	MB
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CHAPTER 4:

An initial exploration of proteomic biomarkers in NASH

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Analysis and interpretation of data	MB
Drafting of chapter	MB
Critical revision	QMA

CHAPTER 5:

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Analysis and interpretation of data	MB
Drafting of chapter	MB
Critical revision	QMA

CHAPTER 6:

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Analysis and interpretation of data	MB and MZ designed and MB optimised primers for pyrosequencing the selected DMRs

	BY extracted ccfDNA from NAFLD and HBV samples and performed pyrosequencing analysis MB extracted ccfDNA from scleroderma samples and performed pyrosequencing analysis MB interpreted results
Drafting of chapter	MB
Critical revision	QMA, JM

CHAPTER 7:

Development of DNA Methylation fibrosis progression signature in NAFLD

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Analysis and interpretation of data	Ashwin Sivaharan (AS) performed bioinformatic analysis of WGBS data MB interpreted results
Drafting of chapter	MB
Critical revision	QMA, JM

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DECLARATION

I confirm that the material and the data used within this thesis were based principally on research performed at Newcastle University and KoC University, Turkey, with contributions from sites which are members of the EPoS research consortium.

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PUBLICATIONS AND PRESENTATIONS ASSOCIATED WITH THIS THESIS

ORAL PRESENTATIONS

- American Association for the study of liver disease (AASLD) and British Association for the study of liver disease (BASL): Development and Validation of The Collagen Neo-Epitope Biomarker Pro-C3 "FIB-C3 Score" For Detection and Staging of Advanced Non-Alcoholic Fatty Liver Disease in A Large International Multi-Centre Patient Cohort (2017)

POSTER PRESENTATIONS

- EASL: Further delineation of fibrosis progression in NAFLD: evidence from a large cohort of patients with sequential biopsies (2016)
- EASL: Simple non-invasive fibrosis scores identify patients with NAFLD who progress to advanced fibrosis/cirrhosis: evidence from a large cohort of patients with sequential liver biopsies. (2016)
- BASL: Development and Validation of The Collagen Neo-Epitope Biomarker Pro-C3 "FIB-C3 Score" For Detection and Staging of Advanced Non-Alcoholic Fatty Liver Disease in A Large International Multi-Centre Patient Cohort (2017)

PUBLICATIONS

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- Boyle, Marie et al. Performance of the PRO-C3 Collagen Neo-Epitope Biomarker in Non-Alcoholic Fatty Liver Disease. JHEP Reports, Volume 1, Issue 3, 2019, Pages 188-198
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BOOK CHAPTER

- Boyle, M. Anstee, Q. Non-Alcoholic Fatty Liver Disease. Evidence based Clinical Gastroenterology and Hepatology. 4th Edition

CHAPTER 1.

INTRODUCTION AND BACKGROUND

1.1. NAFLD Definition

Over the last two decades, NAFLD has become the most prevalent causes of chronic liver disease. The 2016 ***EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease*** defines Non-alcoholic fatty liver disease (NAFLD) as "excessive hepatic fat accumulation, associated with insulin resistance (IR)". It is defined by "either the presence of steatosis in >5% of hepatocytes according to histological analysis or by a proton density fat fraction >5.6% assessed by proton magnetic resonance spectroscopy or quantitative fat/water selective magnetic resonance imaging (MRI)"(1). The diagnosis of NAFLD requires the exclusion of "both secondary causes and of a daily alcohol consumption >30 g for men and >20 g for women" (2). NAFLD includes two pathologically distinct conditions: non-alcoholic fatty liver (NAFL) - simple steatosis and non-alcoholic steatohepatitis (NASH); the latter covers a wide spectrum of disease severity involving fatty infiltration plus inflammation, hepatocellular ballooning degeneration, fibrosis and ultimately cirrhosis. The definitive diagnosis of NASH requires a liver biopsy (1).

1.2. NAFLD Epidemiology

The asymptomatic nature of NAFLD and the lack of a sensitive and specific diagnostic modality other than liver biopsy constitutes a major challenge for large scale incidence and prevalence study execution. Researchers are aware that these public health metrics are frequently underestimated in the general population. ***Prevalenc***e of NAFLD varies based on demographics, the selected diagnostic methodology and disease epidemiology (3-7). The global prevalence of NAFLD is estimated at 25% (95% CI: 22-29) (8), specifically in North America the prevalence range was found to lie between 27% and 34% (9-11) with marked inter-ethic variation (12-14). In Europe, the prevalence is estimated at 25% and varies by region. (15-17) and in Asian countries it is estimated to be between 15% and 20% (17-23). High prevalence of NAFLD has been reported in certain subpopulations. For example, in the morbidly obese, NAFLD prevalence was found in the range of 73-97% and NASH prevalence in the range of 25-33%.(24, 25). Data related to the true ***incidence rate*** of NAFLD remains scarce and fragmented. However, one can speculate, using adult obesity as a surrogate marker for obesity related NAFLD that

since obesity levels have increased approximately two-fold since the early 1960s, NAFLD will likely display a similar trend (26, 27). Reports have estimated NAFLD incidence at 29 cases per 100 000 person-years in the United Kingdom, whereas annual incidence rates in Asia have been reported in the range of 3-5% (28, 29) The prevalence of NASH globally is lower than NAFLD and has been conservatively estimated to be between 2% and 3% (30). The increasing trend of NAFLD is also observed in the paediatric population (31). Paediatric NAFLD prevalence, derived from autopsy data is estimated at 9.6% in 2-19 year olds. In a cross-sectional study involving 41 obese adolescents undergoing gastric bypass surgery, the prevalence of NAFLD was reported at 83%, with 20% having associated NASH. 3-5% of children with NAFLD may progress to cirrhosis (32, 33)

1.3. Brief overview pathogenesis of NASH

A detailed discussion of the specific pathogenesis of NAFLD is beyond the scope of this thesis. However, an overarching review of studies in this area describe the 'steatosis to steatohepatitis transition' as being characterised by mitochondrial dysfunction and increasing hepatocellular oxidative stress (34-36). Other well validated contributory factors are shown in ***figure 1.1*** and include processes such as endotoxaemia; where gram-negative bacteria amongst the gut flora enter the portal circulation due to increased gut permeability and subsequently provoke inflammatory processes (37-39). In addition, there is also some newly emerging evidence to suggest that the bacterial flora within the gut may contribute to the pathogenesis of NAFLD through endogenous alcohol production (40). Supporting this hypothesis, hepatocytes from young patients with NASH have been found to express genes encoding alcohol degradation pathways despite patients abstaining from alcohol (41). In NASH livers, Increased gene transcription of alcohol dehydrogenase (ADH) genes, genes for catalase and cytochrome P450 2E1, and aldehyde dehydrogenase genes was reported showing augmented activity of all the available genes in alcohol catabolism pathways.

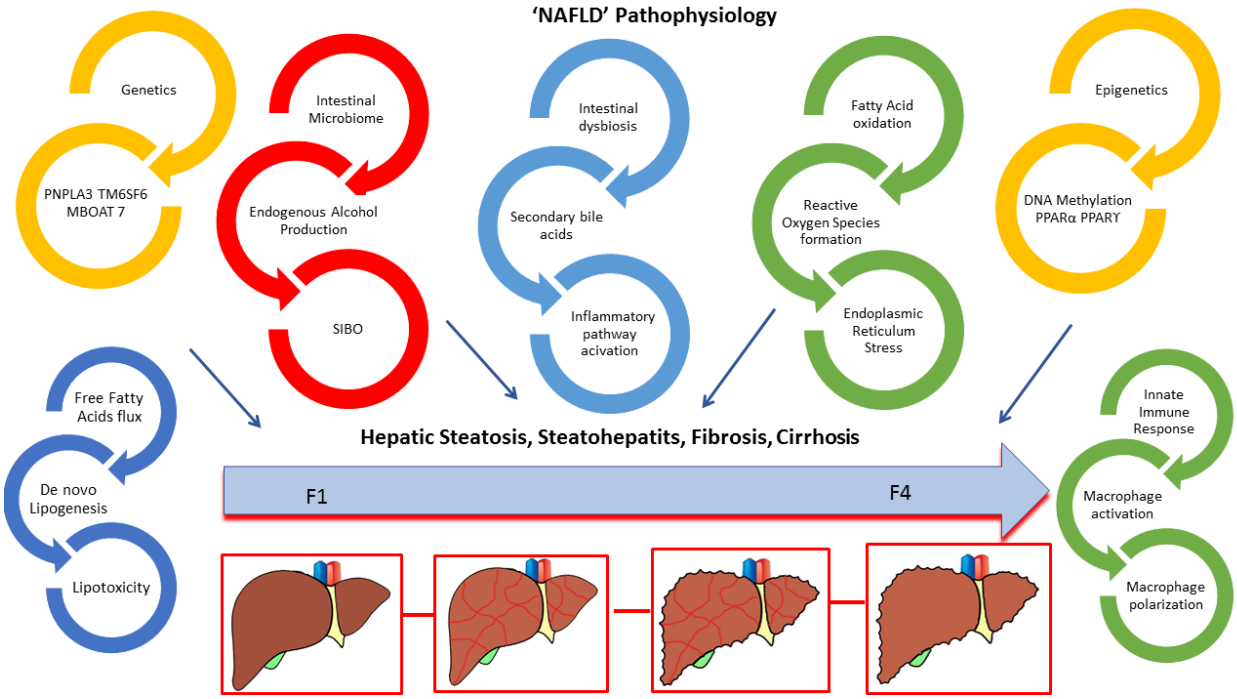


Figure 1.1 NAFLD Pathogenesis

Factors contributing to NAFLD pathogenesis include Free Fatty Acids (FFA) oxidation, reactive oxygen species, endoplasmic reticulum stress, genetics/epigenetics, the innate immune system, gut derived endotoxins, endogenous alcohol, intestinal dysbiosis and small intestinal bacterial overgrowth (SIBO) (42)

1.4. NAFLD Histopathology

Pathological hallmarks of NAFLD include steatosis +/- steatohepatitis. Liver biopsy tissue is required to definitely diagnosis NASH, quantify disease activity and assign fibrosis stage (43). ***Steatosis*** is the accumulation of both micro and macrovesicular hepatic triglycerides droplets in hepatocyte Zone 3 in a diffuse/panacinar distribution (44). It is semi-quantitatively graded by the percentage of liver parenchyma containing steatotic hepatocytes: 0-33%, 33-66%, or >66% (45). **Steatohepatitis**; the histological diagnostic criteria for NASH includes in addition to steatosis, hepatocellular injury and lobular inflammation, usually occurring in zone 3, with or without fibrosis (44) *Hepatocellular injury* is represented by balloon degeneration (enlarged hepatocytes, rarefied cytoplasm +/-Mallory-Denk bodies) with immunohistochemical loss of the normal distribution of keratins 8 and 18. (46). *Lobular inflammatory infiltrates* (lymphocytes,

macrophages, eosinophils and occasional neutrophils) localised as portal inflammation also occur in varying degrees in NASH (47). *Fibrosis* development is described in stages. Initially, it is peri-sinusoidal (acinar zone 3) (48) and with disease advancement, the scarring progresses to bridging fibrosis and cirrhosis (48). Other notable histological lesions include the *ductular reaction*, the presence of *hyperplastic ductular structures* and *connective tissue* at the portal tract interface hypothesised to arise from hepatic progenitor cells and relate to portal and advanced fibrosis (49). ***Grading and Staging NAFLD*;** Reference is made to the semi-quantitative evaluation of NASH associated lesions described by Brunt et al. with the introduction of the concepts relating to 'grading' disease activity and 'staging' fibrosis. Inter-observer variability between pathologists is frequently reported (50), and although other factors are at play, the histopathological criteria used by different groups can be a contributing factor (51).

1.5. Natural History of disease

Despite the large incidence and prevalence associated with NAFLD, a more recent meta-analysis has shown that only a minority of NAFLD patients progress beyond steatosis to develop significant fibrosis or morbidity (52). If patients exhibit progressive disease, a slow rate of progression is the norm, however, there is marked inter-patient variability. The UK DELTA cohort comprises 108 patients with paired liver biopsies at a median interval of 6.6 years reported fibrosis progression in 42% of patients and fibrosis regression in 18% of patients. However, this study also reported a small number of patients experiencing rapid progression to advanced fibrosis i.e. from stage F0 to stage F3-4 over a mean follow-up period of 5.9 years. (53). As the number of paired biopsy studies increased, it is now accepted that the development of progressive liver disease occurs in the settings of both NAFL and NASH, with fibrosis progressing more aggressively in NASH than in NAFL (progression of one stage over 7.1 vs. 14.3 years, respectively (54)). The most recent NASH meta-analysis confirms this trend recording a fibrosis progression rate and a mean annual rate of fibrosis progression in NASH at 40.76% (95% CI: 35-47) and 0.09 (95% CI: 0.1-0.12), respectively (8). Prospective cohort study data suggests that the presence of fibrosis in general, and in particular more advanced fibrosis,

predicts liver-related and all-cause mortality more closely than severity of steatohepatitis as assessed with the NASH Activity Score (NAS) (50, 55, 56). However, the risk of progression to cirrhosis, and subsequent hepatic decompensation remains relatively low. Data from a Danish clinical follow-up study reported an incidence of 3.1% for these end-stage points over a mean follow-up period of 7.6-year (57). These low progression rates were confirmed in longer duration studies with the risk of progression to cirrhosis in NAFL patients estimated at between 0–4% (58, 59). In contrast, estimates of progression to cirrhosis in NASH patients varies with 10% developing decompensated liver disease over 13-years and 25% developing cirrhosis over nine years (60). The rate of progression is influenced by the underlying fibrosis stage and metabolic factors (61, 62). Once cirrhosis has developed, the risk of developing a major complication of portal hypertension is 17%, 23%, and 52% at one-, three- and ten-years, respectively (63). The median survival of patients with cirrhosis once decompensation occurs is approximately two years (64). ***Hepatocellular carcinoma*** Compared to viral liver disease, the annual incidence of hepatocellular carcinoma (HCC) in patients with NAFLD is lower (65). However, given the high global prevalence of NAFLD, NAFLD HCC is now the second most common aetiology of HCC in the US. It is associated with a shorter survival time, principally as it occurs later, with more metabolic co-morbidities contributing to a higher primary liver cancer related mortality. Of clinical concern is the fact that NAFLD HCC can occur in the absence of advanced cirrhosis, presenting problems with regard to effective HCC screening (25, 33, 66-68). The cumulative incidence of HCC in NASH cirrhosis ranges between 2.4% and 12.8% over a 3.2-7.2-year period with a cumulative associated mortality level of 0%-3% over 5.6-21 years (33, 69)

1.6. Morbidity and Mortality Data

NAFLD is the most frequent aetiology of deranged liver function tests in the Western world, reflective of the global obesity and insulin resistance pandemics (70, 71). A diagnosis of NAFLD confers an overall increased mortality compared to the general population (72); with a concurrent diagnosis of NASH associated with a >10-fold likelihood of liver related death (2.8% vs. 0.2%). The most common causes of death in this population are cardiovascular disease and malignancy (60, 73)

1.6.1. Extrahepatic Complications of NAFLD

(1) Metabolic syndrome; The reported prevalence of metabolic syndrome in the NAFLD population is 42.54%; 95% (CI: 30--56); with individual metabolic syndrome component prevalences as follows; obesity (51.34%; 95% CI:41-61), type 2 diabetes (22.51%; 95% CI: 18-28), hyperlipidaemia (69.16%; 95% CI: 50-83) and hypertension (39.34%; 95% CI: 33- 46) (8) NAFLD is therefore justifiably considered as the hepatic manifestation of this syndrome (74, 75). A bidirectional relationship exists between NAFLD and T2DM with NAFLD promoting the development of T2DM and vice versa (52, 76, 77). The dual diagnosis both of metabolic syndrome and NAFLD immediately identifies patients at higher risk for overall morbidity and cardiovascular related death (78).

(2) Cardiovascular disease. There are consistently reported strong associations between NAFLD, endothelial dysfunction, arterial stiffness, hypercoagulability status, coronary artery atherosclerosis, cardiac and valvular function, congestive heart failure and arrhythmias (79-86). The most important clinical implication of these associations is an increased cardiovascular mortality observed in the NAFLD population, independent of traditional risk factors where NASH is associated with a doubling of cardiovascular risk (60).

(3) Chronic Kidney Disease (CKD). NAFLD is associated with an approximately two-fold increased risk of CKD where studies have found a correlation between NAFLD and CKD after adjustment for co-morbidities (87-89). Over the past 5 years the incidence of simultaneous liver-kidney transplantation has increased exponentially (90). In the US, the United Network Organ Sharing (UNOS) database during the years 2002–2011, recorded that 35% of patients transplanted for NAFLD related cirrhosis progressed to stage 3b-4 CKD within two years after liver transplantation in comparison to 10% of patients transplanted for other aetiologies (91).

(4) Malignancy The second most common cause of mortality among NAFLD patients is extrahepatic malignancy (92). The colon has been identified as the extrahepatic site where this link has been most consistent, with a significant proportion of malignant lesions developing in the proximal colon at a much younger age (93).

1.6.2. Disease associations

(1) Polycystic Ovarian Syndrome (PCOS); Women with PCOS exhibit a 4 fold increased rate of NAFLD compared to healthy controls (94). This observation requires clinical attention as NASH with varying degrees of fibrosis has been reported among young women with PCOS who may not otherwise be screened for advanced disease (95)

(2) Obstructive Sleep Apnoea (OSA) Obstructive Sleep Apnoea, (OSA) is found to effect 35-40% of patients that are obese and is a risk factor for NAFLD (96). Several studies have found association of OSA with advanced NASH histology (97-99)

1.7. Patient-reported and economic Burden of NAFLD as an adjunct to morbidity and mortality data

The previous discussion testifies that an informative body of literature exists relating to NAFLD morbidity and mortality. The take home message regarding liver-specific mortality and overall mortality among NAFLD and NASH are figures of 0.77 per 1,000 and 11.77 per 1,000 person-year and 15.44 per 1,000 and 25.56 per 1,000 person-years respectively; alongside NAFLD incidence risk ratios for liver-specific and overall mortality reported at 1.94 and 1.05 respectively (8) Scalable data on NAFLD QoL and economic burden is not as readily available. Currently no pharmacological therapies for NAFLD exist. However, several drugs are currently in phase III clinical trials, with FDA approval imminent. In 2019, there now exists a limited window of opportunity to record data on patient reported outcomes (PROs) and the economic burden of NAFLD in anti-fibrotic treatment naïve populations. Evaluations in these fields will complement the mass of morbidity and mortality data currently available and allow clinicians to critique new treatment regimens beyond a purely clinical perspective to assess the full benefit of NASH treatment.

1.7.1. ***Patient Reported Outcomes (PROs) in NAFLD*;** A limited number of studies have consistently shown significant impairment of patient PROs in NAFLD, specifically those assessing HRQL. Pursuit of this as a research objective is supported by the observation

that in HRQL assessments, patients with NAFLD score lower relative to patients diagnosed with other chronic liver diseases (100-103).

1.7.2. ***Economic burden in NAFLD***; In the US, complex economic models estimated the annual direct medical costs of NAFLD to approximate $103 billion ($1,613 per patient). Applied to Europe, the models projected an annual cost of about €35 billion (from €354 to €1,163 per patient). The financial burden associated with NAFLD is likely to be considerable and to date, this analysis has not been explored in detail in the United Kingdom. This data will be valuable when deriving cost-benefit analysis data on new anti-fibrotic treatments.

1.8. UK NAFLD Quality of Life (QoL) Burden

Current clinical trials focus on surrogate markers for histological endpoints. However, true endpoints as defined by the FDA, involve improvement in more domains , to include how the patient "feels and functions" in addition to "survives" (104). In this thesis, individual QoL and patient reported outcome (PRO) measures in addition to the health system economic burden in NAFLD will be considered. A brief, current literature review in these areas is summarised below.

1.8.1. Patient Reported Outcomes (PROs)

The Food and Drug Administration [FDA] define patient reported outcomes (PROs) as "any report of the status of a patient's health condition that comes directly from the patient, without interpretation of a patient's response by a clinical" (105). PROs are developed with input from clinicians, patients and psychometric experts alike and as such PRO endpoints have the potential to add value to clinical trial interpretation. Indeed, a recent review of the FDA labels has shown that 71% of FDA approved products included a PRO as a primary trial endpoint (106). PRO inclusion ensures that the impact of a trial intervention is comprehensively evaluated (beyond simply biochemical/histological outcomes alone) and are also valuable as secondary endpoints

to aid the interpretation of primary endpoints. They can provide useful information pertaining to regulatory decisions, cost-effectiveness analyses and informing clinicians to select best treatments (prioritising patients for life-style interventions or pharmacological interventions) and inform health policies (107).

1.9. Economic Burden of NAFLD

1.9.1. Burden of chronic liver disease on health care resources

Liver disease mortality rates have increased 400% since 1970 with a high disease burden caused by obesity (108). Obesity costs the NHS 6.1 billion per year with a loss of productivity of 5.6 billion over 2 years (109). The recent EASL endorsed HEPAHEALTH exposed Europe as having the largest burden of liver disease in the world (110). Worryingly, the UK exhibits a liver disease growth rate exponentially higher than other countries in Western Europe and currently no real-world data on the burden of NAFLD on health-care resources is available from European countries. NAFLD costing data from a small number of US studies is available but it is important to appreciate the inherent diversity in liver disease epidemiology and that variations in disease aetiology and risk factors are country specific (111, 112).

1.9.2. Specific burden of NAFLD Health care utilisation in outpatient settings

A recent US study of 29,528 patients who were referred for outpatient care for NAFLD over a 5-year period reported a doubling in the number of referrals (3585-6646); concurrent with an increase in annual costs attributable to the increase in the number of OPD visits and the increasing frequency of co-morbidities in this population (111). Worldwide, NAFLD is the most common cause of chronic liver disease (8). This study aims to record the prevalence in tertiary liver clinics of NAFLD versus other reasons for medical consultation. It is critical to describe the utilization of medical resources triggered by a suspected NAFLD diagnosis to identify future obstacles in delivering effective care. This objective often loses momentum in public health forums as chronic

liver disease (CLD), particularly NAFLD has a considerable latency period during which affected individuals are asymptomatic, despite the development of hepatic fibrosis. Currently, the Lancet commission in the UK require 'real time' data provided by this type of study to justify an increase in provision of medical and nursing training in hepatology to cope with increasing service demands (113)

1.10. Problems Studying NAFLD Disease

While studies to elucidate NAFLD natural history and pathogenesis have been impressive to date, research in this field is challenging and affirms NAFLD as an archetypal complex trait.

1.10.1. Experimental Models of NAFLD in the study of human disease

The bank of epidemiological and natural history data in NAFLD is derived from observational studies. To fully explain these observations, it is necessary to translate these hypotheses from "bedside to bench" at a basic science level. Unfortunately, an animal model of NASH that perfectly represents human disease is not available. To simulate a physiological comparable model, rodent metabolic profiles are being manipulated by being fed "combination" diets with dual obesogenic/insulin resistance properties with anti- obesogenic/insulin sensitizing diets that are more fibrogenic. This then necessitates that any novel finding uncovered in animal models to undergo validation in human tissues, making interpretation of novel research finding slow and laborious. However, in this thesis as a result of well-developed research consortia in NAFLD such as EPoS and LITMUS, collaborative bio-banking efforts have permitted molecular profiling of well-characterized human samples as an alternative or complementary avenue to the study of NAFLD human disease. In the pre-clinical stages, this expansive research platform can be used to guide work in refined animal models. This has consequently revolutionised current approaches to research in NAFLD and the future is optimistic with regards to gaining increased insights into NAFLD disease progression (104).

1.10.2. Problems studying NAFLD in clinical settings

NAFLD observational studies are challenging. Validation of research hypotheses in NAFLD require robust diagnostic criteria defining the target disease. However, in NAFLD, research findings are oftentimes criticised owning to diagnostic uncertainty in disease grading and staging whereby researchers are limited by imperfect diagnostic modalities. When assessing patients with NAFLD, the key clinical issues are: (I) to differentiate NAFL from NASH; and (II) to determine the stage of fibrosis. Liver biopsy remains the established but imperfect reference standard for investigation being invasive, resource intensive, prone to sampling error and carrying a small but significant risk of complications (114). It remains impractical outside specialist practice, and unsuitable when a large 'at risk' population needs to be assessed (115). A review of the diagnostic modalities employed in NAFLD are summarised below.

1.10.3. The current reference standard: Liver Biopsy

Histological evaluation remains the "gold" standard to assess NAFLD patients (116). However, widespread use of liver biopsy as a diagnostic tool is impractical (117-119). 84% of patients experience mild pain after a liver biopsy; with more severe pain, major bleeding, infection or death occurring in approximately 0.3% of cases (120). However, currently there is no competing investigation providing simultaneous information on steatosis, inflammation, hepatocellular injury, fibrosis, response to treatment and concurrent liver disease. Most expert guidelines continue to recommend liver biopsy for NAFLD patients at high risk for NASH and/or advanced fibrosis, if there are discordant non-invasive tests or if NAFLD is suspected as a co-existing chronic liver disease (121-123). However, in the real world, the ever-increasing prevalence of NAFLD mandates a shift from histology towards the development of non-invasive assessments (33)

The above challenges impact particularly in the clinical investigation of potential new anti-fibrotic therapies and routine clinical management. The current drug development regulatory pathways to registration require demonstration of histological improvement in NASH (124). This obliges potential clinical trial participants to undergo an initial

'screening' liver biopsy with investigator sites reporting screen failure rates as high as 70% (125). Liver biopsies may be liable to sampling error with consequent misdiagnosis and incorrect grading and staging of disease (114). A study involving 51 patients who underwent percutaneous liver biopsy (2 samples collected) reported substantial agreement in steatosis grade only (56). Moderate and slight agreement were demonstrated for hepatocyte ballooning/peri-sinusoidal fibrosis and lobular inflammation respectively (114). Single sample collection resulted in missed ballooning detection in 24% of samples. Concordance of results between 2 samples was reported as higher for fibrosis than for inflammation, however, a 41% fibrosis discordance rate of at least one stage was recorded with 12% of subjects demonstrating no /mild fibrosis on one sample and bridging fibrosis on the other. Subsequent studies have suggested that when considering clinical trials endpoints, sampling error is less pronounced when the endpoint involves a change in the staging score of 2 or more points rather than the resolution of fibrosis (114). This level of diagnostic uncertainty complicates the study of NAFLD and to ameliorate this, at the very least, liver biopsies should be reported by an experienced histopathologist using a validated histological scoring system such as the NASH-CRN Score (45) or the SAF Score (126). However, this approach is also suboptimal as demonstrated in serial publications by Kleiner et al (2005 and 2019) involving the same group of 9 experienced histopathologists(45, 127). In the 2005 publication validating the NASH-CRN score the k-statistic for inter-rater agreement was 0.84 for fibrosis, 0.79 for steatosis, 0.56 for injury, and 0.45 for lobular inflammation. Agreement on diagnostic category was 0.61. In the 2019 publication, values were 0.75 for fibrosis, 0.77 for steatosis, 0.54 for injury, 0.46 for lobular inflammation and 0.66 for steatohepatitis diagnosis. Disappointingly, no improvement in inter-observer agreement was observed over a 15-year period.

1.11. Steps to Improve Care Delivery

Optimal therapy development will be dependent upon efficient diagnostic modalities to improve patient ascertainment in routine practice, optimise clinical trial recruitment

and efficiently prognosticate to decide who is at greatest risk and may therefore derive the greatest benefit from therapy.

Currently, the optimal clinical management of NAFLD is challenged by uncertainties in diagnostic testing (NAFLD is frequently asymptomatic), risk stratification (NASH with advanced disease may be present even in those with relatively normal clinical biochemistry) and how best to monitor affected patients for disease progression. Given the long natural history of NASH and the frequent comorbidities in this population, studies designed to show improvement in survival or quality of life measures may be difficult to conduct (104). Although steatohepatitis is widely considered the biological driver of disease progression, fibrosis is the only histological feature that has been found to be independently associated with long-term prognosis. Advanced fibrosis stage confers an increased risk of progression to cirrhosis, liver failure and HCC and is associated with an increased likelihood of all-cause mortality (53, 54, 56, 128). Currently, resolution of NASH (without worsening fibrosis) or reduction of fibrosis stage (without worsening NASH) are the accepted endpoints by the regulatory authorities (129). There are no "gold standard" endpoints that can be followed in lieu of histology (such as monitoring of viral load in trials of hepatitis C and hepatitis B), liver histology currently remains the main outcome variable as an endpoint for clinical trials, although other measures such as MRI PDFF, magnetic resonance elastography are increasingly being used in Phase 2 studies (104).

The remainder of this thesis focuses mainly on fibrosis diagnostic and prognostic biomarkers, with lesser considerations given to other NASH parameters.

1.11.1. Personalised Health Care

In line with the objectives of this thesis is the FDA move towards Personalised Healthcare Care (PHC). PHC recognises the need for non-invasive diagnostic and prognostic biomarkers in NAFLD. Essential elements of PHC include identification of

optimal treatments and a responsive target population. An important challenge in NAFLD lies in its well described clinical heterogeneity. Currently there are no convincing biomarkers to stratify progressors versus non-progressors and slow or fast progressors as in addition to reliably grading fibrosis stage in NAFLD patients, there is a clinical need to identify patients that are likely to progress at earlier stages to prevent fibrosis progression and arrange suitable follow-up. Given the size of the at-risk population, expensive or invasive tests will not be suitable for this purpose. Circulating biomarkers, originating from defined fragments of scar tissue may serve as valuable tools for precision medicine. Cirrhotic livers are reported to contains up to 10 times more collagen than a healthy liver (130), therefore a biomarker panel comprising of well characterised collagen formation and degradation products may allow rapid assessment of fibrosis stage, efficacy of treatment with anti-fibrotic therapies and lead to the development of a personalised therapeutic approach. OMICs data also harbours equally exciting opportunities.

1.11.2. Need for therapeutic agents

A motivator for NASH research is the fact that NASH represents an unmet medical need where a deficiency of FDA approved therapies exists. Commonly implemented interventions at present are lifestyle related. Weight loss is effective for fibrosis reversal; however, it is uncommon for subjects to achieve and sustain the 7-10% decrease needed to see histological improvement and bariatric surgery remains an unlikely solution for the target population (131-133). Currently there are >700 NAFLD clinical trials registered on clinictrials.gov, with 53 Phase III studies (134-139).

1.11.3. Drug Treatment of NAFLD

As previously mentioned, no FDA approved pharmacological therapies are available in NASH. However, some existing medications have been repurposed to treat NASH(140), namely Vitamin E and Pioglitazone. Pioglitazone improves components of the NAS score, however is associated with an increased risk of bladder cancer and MI(54, 141, 142).

Vitamin E is associated with improvement in histological lesions, however may increase overall mortality (143, 144), therefore each agent must be considered on a case by case basis. New therapies with strong experimental evidence are currently being trialled in human NASH, and are summarized in table 1.1. (145). Obetacholic acid (OCA) (REGENERATE phase 3 study) is the most promising agent to date and is one of the first agents associated with robust, beneficial changes in liver histology. The endpoint for the improvement in fibrosis was achieved by 37 (12%) patients in the placebo group, 55 (18%) in the OCA 10-mg group, and 71 (23%) in the OCA 25-mg group (146). This agent is in contrast to Elafibranor (RESOLVE-IT phase 3 study), which did not demonstrate a statistically significant effect on the primary endpoint of NASH resolution without worsening of fibrosis and was suspended by GENFIT in May 2020**.** Currently, there are about 196 agents being evaluated for the treatment of NASH, with many phase 2 and 3 trials ongoing. Development of non-invasive fibrosis and NASH biomarker will be pivotal to improve clinical trial recruitment and retention numbers.

Table 1.1 NASH drugs currently in phase 2 and 3 clinical trials

Drugs	Mechanism of action	Phase in clinical trial	Trial identification
Obeticholic acid	FXR agonist	III	NCT02548351
Cenicriviroc	CCR2/CCR5 inhibitor	III	NCT03028740
MSDC-0602K	MPC inhibitor	IIb	NCT02784444
NGM282	FGF19 analogue	IIb	NCT03912532
Saroglitazar	PPAR-α/γ agonists	II	NCT03061721
Resmetirom	THR-ß agonist	III	NCT03900429
Tropifexor	FXR agonist	IIb	NCT02855164
Aramchol	SCD1 inhibitor	III	3rd quarter 2019
Selonsertib	ASK1 inhibitor	III	NCT03053050

1.11.4. Clinical Trial Challenges

Anti-fibrotic agent approval has been hampered by many factors. The histological and clinical end-points stipulated at the onset necessitates that NASH clinical trials are long-term with large patient numbers, necessitating multiple liver biopsies in the absence of approved biomarkers. Biomarkers to date for steatohepatitis in NASH have been unsuccessful; however, research perspectives are changing following the CENTAUR trial. This trial demonstrated an agent with independent anti-fibrotic activity and challenged the assumption that the anti-fibrotic effects of NASH agents could just target fibrosis without affecting the histological features of steatohepatitis at 1 year further increasing the interest in fibrosis biomarkers for clinical trial monitoring (139).

1.11.5. Over-reliance on sub-optimal screening tools

Current significant challenges in clinical trial recruitment are demonstrated in the differences observed in patient recruitment rates in the US versus Europe. Study investigators have speculated that over-reliance on non-invasive screening techniques and the development of algorithms incorporating Fibroscan™ (vibration controlled transient elastography, VCTE). In Europe, Fibroscan™ is often used as a diagnostic tool therefore these sites have lower rates of liver biopsy. In this context, rather than reconfigure European attitudes to Fibroscan™ being helpful as opposed to diagnostic, experts in the field have proposed exhaustive validation of "wet" blood based biomarkers to pre-screen patients more comprehensively and so enrich the pool of potential trial participants(147).

1.12. Current status: Non-Invasive evaluation of Liver Disease

As previously mentioned, NASH is a histological entity. An objective in the research world therefore remains to develop a non-invasive NASH biomarker. Over the last decade, there have been potential practice changing developments in imaging modalities (Magnetic Resonance and Transient Elastography) to diagnose and quantify

steatosis and fibrosis. However, advances to noninvasively diagnose NASH and quantify disease activity have not experienced such successful innovation.

1.12.1. *Diagnosis of NASH versus Hepatic Steatosis* (33).

In NAFLD, clinical goals involve (1) Quantification of steatosis (2) Discriminating NASH versus NAFL and (3) determining fibrosis stage. A plethora of biomarkers and clinical models have been proposed to discriminate NAFL from NASH ***(Table 1.2)*** but none have so far been sufficiently accurate for use in routine practice (115, 148). The diagnostic accuracies of these markers, indicated by the areas under the receiver–operating curve (AUROC), are in the range of 0.70 to 0.90. However, lack of external validation, reproducibility and availability preclude their use as stand-alone tests in clinical practice (149, 150). Possible NASH biomarkers range from first principle alanine aminotransferase (ALT) levels and metabolic syndrome to more specialist circulating keratin18 fragment levels (CK-18). Regarding ALT levels (151, 152), a cut-off of <35 U/L had recorded NASH levels of 11% versus 29% at a cut-off ≥35 U/L. Broader parameters involving a cut-off level of ALT 2X ULN (>70 U/L) had 50% sensitivity and 61% specificity for predicting NASH obviously limiting its clinical utility. The most promising but flawed NASH biomarker to date was CK18 described by Feldstein et al, but limited sensitivity and specificity curtailed its widespread adoption (153). However, now the NAFLD scientific community have openly cast aspersions on the tractability of NASH resolution in clinical trial settings. Recent publications have suggested that NASH is a transient, dynamic state and that change in fibrosis stage may be a more tractable clinical trial endpoint. A non-invasive test with the objective to identify both NASH with stage ≥2 fibrosis, which is the sub- phenotype that is primarily targeted in Phase 2B and Phase3 clinical trials at present now represents a more clinically useful biomarker target.

Table 1.2 Diagnosis of NASH versus Hepatic steatosis Reference (33)					
Test	**Description**	**AUROC**	**Sensitivity**	**Specificity**	**Routine use**
Cytokeratin-18 (CK-18) (153, 154)	CK-18 fragments from apoptotic hepatocytes	0.65	58	68	Limited sensitivity
PIIINP(155)	Amino terminal peptide of type III procollagen, released from the precursor peptide during the synthesis and deposition of type III collagen	0.82-0.84	80	73	Not externally validated. Trials small numbers
Predictive Models					
Nash test (156)	Age, sex, height, weight, serum triglycerides, cholesterol, alpha2macroglobulin, apolipoprotein A1, haptoglobin, gamma-glutamyl-transpeptidase, transaminases ALT, AST, bilirubin	0.79	33%	94%	Not externally validated
NASH Diagnostics (157)	CK-18, adiponectin, resistin	0.73	71.4%	72.7%	Not externally validated
Nice Model (158)	ALT, CK-18, presence of MetS	0.88	84%	86%	Not externally validated
HAIR (24)	Hypertension, ALT, IR	0.90	90%	89%	Not externally validated
oxNASH (159)	13-hydroxyl-octadecadienoic/linoleic acid ratio, age, BMI and AST	0.83	81%	97%	Not externally validated
NASH Score (160)	*PNPLA3* genotype, AST, fasting insulin	0.73-0.77	0.55-1	0.7-1	Not externally validated
Palekar Score (161)	8-epi-PGF(2alpha), TGF-beta, adiponectin, and hyaluronic acid(HA)	0.763	73%	65%	Not externally validated
Imaging Techniques					
Although imaging is useful in detecting steatosis, it no established techniques can distinguish NAFL vs. NASH or stage fibrosis (115, 162). Numerous experimental studies explored different imaging modalities to differentiate NAFL from NASH but there are too few studies in humans to draw any conclusions (163)					

1.12.2. Diagnosis and Staging of Hepatic Fibrosis (33)

Serological markers for the evaluation of liver fibrosis can be divided into 'indirect' markers (that reflect alterations in hepatic function but not collagen turn-over, e.g. platelet levels) and 'direct' markers (associated with fibrogenesis/fibrolysis) (115) ***(Table 1.3***). Alone, routine clinical blood tests are not reliable surrogate markers for fibrosis but are helpful when combined with other indices as part of 'indirect marker' panels (e.g. AST/ALT Ratio, FIB-4 score or NAFLD fibrosis score (NFS)). These diagnostic panels exploit the premise that ALT falls whereas AST remains stable or increases as disease progresses towards cirrhosis. The consequent increase in the AST/ALT ratio is a component of many of the simple panels. These tests have moderate specificity and therefore a limited positive predictive value but a high sensitivity and so good negative predictive value for advanced fibrosis especially when applied to populations where the pre-test probability is quite low, even when the ALT is within the normal range ***(Table 1.3)*** (164, 165).

The NAFLD Fibrosis Score (NFS); by applying a low cut-off (<-1.455), advanced fibrosis can be excluded with high accuracy (NPV 93%) whilst a high cut-off threshold (>0.676) offers accurate detection of advanced fibrosis (PPV 90%). Use of this score has been suggested to reduce the need for liver biopsy by ~75%. The ***FIB4 Score*** is currently cited as the best performing simple non-invasive tests for advanced fibrosis in NAFLD. A score of <1.3 has a 90% NPV for stage 3-4 fibrosis, whilst a score of >2.67 had an 80% PPV with only a quarter of the cohort being unclassified (164).

In patients with risk factors for NAFLD, tests with a good negative predictive value are helpful in excluding cases with a low probability of significant disease (115). However, it is noteworthy that these scores were developed and validated in patients aged between 35 and 65 years of age and perform less well in those aged ≤35-years or >65-years of age. In particular, the specificity of the FIB-4 and NFS decline with age, becoming unacceptably low in those aged ≥65 years (35% for FIB-4 and 20% for NFS), running the

risk of triggering unnecessary investigations in older patients. As a result, new cut-offs have been derived and validated for those aged ≥65 years, which improve specificity to 70% without adversely affecting sensitivity (FIB-4 2.0, sensitivity 77%; NFS 0.12, sensitivity 80%) ***(Table 1.3).***

Other commercial assays are currently in development and detect pathologically modified proteins generated by specific proteases. Specific collagen fragments such as PROC3 and PROC6 may be detected using proprietary Protein Fingerprint ^{TM} ELISA assays and have thus far provided promising results and will be validated as part of this thesis (166). Other promising biomarkers on the horizon are in the field of lipodomics, MicroRNA and epigenetics. However, they require further validation before they can be implemented into clinical practice (167-170).

Routine ***imaging modalities*** (ultrasound, CT and MRI) can accurately diagnose cirrhosis if features such as a nodular appearing hepatic parenchyma, enlarged caudate lobe or signs of portal hypertension are present but are neither sensitive nor specific for detecting intermediate stages of fibrosis (115). A range of more specialised techniques have been developed and are now routinely used in clinical practice. Ultrasound based elastography techniques (e.g. Fibroscan, ARFI, SuperSonic) have been shown to be a safe, quick, and cheap methods to assess for advanced fibrosis and so are more widely adopted although they too have a higher negative than positive predictive value and require disease specific validated cut-offs to be applied. Accuracy of elastography may also be adversely affected by body habitus (171). MR-elastography holds the most promise as a useful fibrosis diagnostic tool, however its implementation is limited by cost.

Non-invasive tests may be used in combination to triage patients for further investigation e.g. serially applied indirect blood markers to complement transient elastography. When there is concordance, no further testing is needed. When discordant, it is reasonable to consider liver biopsy in selected cases.

Table 1.3: Diagnosis and Staging of Hepatic Fibrosis (Serum Biomarkers and Imaging					
Predictive Models for Advanced (F3/4) Fibrosis					
Test	**Component**	**AUROC**	**Threshold**	**Sensitivity**	**Specificity**
Simple Tests					
AST/ALT ratio (164)	AST, ALT	0.83	0.8 1.0	74 52	78 90
AST to platelet ratio index (APRI) (164)	AST, platelet count	0.67–0.94	1.0	27	89
BARD (164, 172)	Body mass index (BMI), AST/ALT ratio, diabetes	0.77	2.0	89	44
FIB-4 (164, 173)	Age, AST, platelet, ALT	0.86	1.30 3.25 Age adjusted lower threshold if > 65 years:2.0	85 26 80	65 98 70
NAFLD fibrosis Score (164, 173, 174)	Age, BMI, hyperglycaemia, platelet, albumin, AST/ALT ratio	0.81	-1.455 0.676 Age adjusted lower threshold if >65 years: 0.12	78 33 77	58 98 70

Advanced Panels					
Test	**Component**	**AUROC**	**Fibrosis Stage**	**Sensitivity**	**Specificity**
ELF test (175)	Age, HA, TIMP-1, PIIINP	0.82–0.90	F2-F4	80	90
FibroMeter (176)	Platelet count, g2 macroglobulin, AST, age, prothrombin index, HA, blood urea nitrogen	0.90–0.94	F2-F4	81	84
FibroTest (177)	Alpha-2 macroglobulin, haptoglobin, GGT, total bilirubin, apolipoprotein	0.81–0.92	F3-F4	15–77	77–90

Hepascore (178)	Age, gender, bilirubin, gamma-glutamyltransferase(GGT), hyaluronic acid, and a2-macroglobulin	0.81 0.90	≥F3 F4	75.5 87	84.1 89

Imaging for Advanced (F3/4) Fibrosis				
Imaging Technique	**Advantage**	**Disadvantage**	**Comments**	**Effective in Fibrosis Detection**
Transabdominal US (179-181)	Widely available; safe; inexpensive; accurate diagnosis of moderate-to-severe hepatic steatosis Sensitivity (81.8 to 100 %) and specificity (98 %)	Limited sensitivity when steatosis <30%	A four-point scale has been developed to assess the degree of steatosis	Unreliable in detection of fibrosis.
CT (Computed Tomography) (182)	Evaluate entire liver structure; Hepatic lesion detection	Expensive; Radiation exposure; Limited sensitivity if steatosis is mild		No role in detection of early fibrosis
MRI Elastography (MRE) (183)	Safe; non-invasive; Analyse entire liver structure; Can be used in obesity and ascites	Cost; Time; Expensive; Not used in livers with iron overload	An external probe emits low frequency vibrations throughout the liver. These vibrations are effected by the amount of fibrotic tissue present in the liver and are measured by MRI spin echo sequence to quantify liver fibrosis	High Sensitivity (85%) and specificity (93%) at a cut-off of 4.15 kPa for the diagnosis of advanced fibrosis (AUROC 0.95)
Transient ultrasound elastography (TE)(FibroScan™) (184-186)	Quick; Painless; Non-invasive; Measures a larger sample of the liver (1/500) compared to, conventional liver biopsy (1/50,000); Accurate; Reproducible; XL probe for obese patients	Results influenced by hepatitis;; liver failure; fatty infiltration; cholestasis; congestion; postprandial increase in	This is an ultrasound-based vibration-controlled elastography (VCTE) technique which quantifies the degree of liver "stiffness", as a function of the extent of hepatic fibrosis	High sensitivity (86.1%) and specificity (88.9%) at a cut off of 5.9kPa for detection of fibrosis (F > 1)

		portal blood flow. Precise validation of specific stiffness cut-off values for the various stages of fibrosis in NAFLD is still lacking.		
Acoustic radiation force impulse imaging (ARFI) (187-189)	Available on most standard US machines; Not effected by the presence of ascites and obesity; equivalent accuracy to TE for the detection of advanced significant fibrosis and cirrhosis	Poor discriminatory power for Intermediate fibrosis stages; Direct comparison with TE is difficult as units are (m/sec) not (kPa); Narrow range of values (0.5-4.4 m/sec)	A real-time B-mode ultrasound image is generated, as a result of a shear wave which has been focused on the high-risk area by the machine operator. This system can be seamlessly integrated to standard US machines as a useful tool to quantify fibrosis.	A histologically based comparative study of fibroscan, ARFI and SSI found that these diagnostic modalities were equivalent in their ability to diagnose mild fibrosis (>F1) or cirrhosis (F4). SSI was found to be better at diagnosing significant fibrosis (>F2) than ARFI and better at detecting advance fibrosis (>F3) than FibroScan
Supersonic shear wave elastography (SSI) (268-270)	Can be integrated on a regular US machine; Region of interest (ROI) can be selected to focus on high risk areas; Higher range of values (2-150 kPa) than ARFI; Accurate for cirrhosis	Poor discriminatory power for Intermediate fibrosis stages; Quality criteria not well defined; Validation necessary to determine applicability	Pulse wave beams emitted by an ultrasound transducer serves to generate a real-time colour mapping of liver elasticity. This is superimposed on the standard B-mode real time image, facilitating quantification of tissue elasticity; a surrogate marker for fibrosis	

1.13. Biomarkers, current status

Liver biopsy remains the established but imperfect 'gold standard' investigation. A lack of tractable non-invasive biomarkers has impeded the diagnosis, risk stratification and monitoring of patients. It has also hampered drug development and the conduct of clinical trials, which still depend on histological effect as an endpoint. NAFLD biomarkers are codified within three FDA BEST biomarker target domains as per ***table 1.4***

Table 1.4. FDA BEST biomarker target domains	
Code	**Purpose**
Diagnostic Markers	To estimate current fibrosis stage
Prognostic Markers	To stratify individuals by fibrosis progression risk, discriminating fast vs. slow progressors and/or predicting long-term outcomes and hard endpoints
Monitoring Markers	To track disease progression or treatment response.

Disease biomarkers may be at one of four qualification levels as ***per table 1.5***

Table 1.5. Biomarker qualification level	
Qualification Level	
Exploration	Early-phase experimental biomarkers
Demonstration	"Probable valid" biomarkers
Characterisation	"Known valid" biomarkers
Surrogacy registerable	"Surrogate endpoint"

Although there has been some progress in biomarker development for detection of advanced fibrosis, existing biomarkers are generally at the first two qualification levels and need validation.

1.14. New Biomarkers Avenues

1.14.1. Role of collagens in organ fibrosis

Progression to cirrhosis is associated with a 10 fold increase in fibrillar collagens (type I, III and V) and up to a 6 fold increase in type VI collagen resulting in increased liver stiffness (190). The space of Disse becomes capillarised, in parallel to a 2 fold increase in ECM elastin concentration (191) which results in ECM architecture disruption (192-194). A non-specific molecular signature of this transition could be

summarised as an increase in type I over type III collagen with a decrease in type IV collagens (195). Studies have advanced and are now reporting that serum/plasma levels of certain procollagen fragments can be used to assess the balance of fibrogenesis and fibrolysis (130).

1.14.2. Circulating collagen fragments

Collagens are trimeric molecules that combine to form robust triple helix structures in the extracellular matrix (ECM). The collagen fragments that will be interrogated in this study belong to the fibril forming collagen subtype (type III), the network forming collagen subtype (type IV) and the interconnecting collagen subtype (type VI) (196). In simple terms, type IV collagen has been described as a 'smart collagen' for tissue repair processes, whereas type III collagen is produced by fibroblasts to maintain their function therefore constitute the 'glue and structure' in tissue repair (130). The dynamic composition and spatial distribution of collagens during fibrosis progression provides an unexplored milieu for biomarker development (130). So far, 28 types of collagen have been characterised and their degradation products in some cases have been shown to be important in biological feedback loops integral in ECM production and fibrosis development (130).

1.14.3. Biomarkers that reflect fibrogenesis and fibrolysis

Fibrillar collagens are synthesised with pro-peptides molecules which undergo proteolytic cleavage before collagens can form triple helices in the ECM. These pro-peptides molecules and their subdomains can be quantified as serum biomarkers for ECM formation, turnover or degradation (193, 197) . 'Protein fingerprint' technology has been exploited in the development of novel biomarkers focusing on the identification of such pathologically modified proteins. In simple terms, fibrogenesis involves protease mediated connective tissue destruction which results in the creation of a unique protein degradation sites i.e. a 'protein fingerprint' which serves as a target for a neo-epitope specific antibody. A number of assays have been developed to quantify collagen fragments reflecting collagen formation (PROC3, PROC6 and PROC4) and degradation (C3M and C4M) in preclinical models and

patients (198, 199). This study will look to validate these fragments as fibrosis diagnostic biomarkers.

1.15. Alternative platforms for biomarker development

In this thesis, we first examined direct biomarkers (Proteomic biomarkers). Proteomic biomarkers are currently being characterised by the FDA and EMA to the extent where FDA approval for inclusion in drug clinical trials is imminent. The data generated in this thesis will be made available for their review. Although the terminology implies that NAFLD is a single disease entity with a spectrum of manifestations, it is more likely a heterogeneous collection of overlapping disorders. In line with this broader appreciation of NAFLD as a disease, it is advisable to consider the whole genome. Liver disease (NAFLD specifically) has benefited from high throughput technologies (genomics, epigenomics, transcriptomics, proteomics and metabolomics). In particular, genomic, epigenomic and transcriptomic data are now being characterised to assess NAFLD progression risk and offers a new forum for biomarker development (200).

1.15.1. Genomics, transcriptomics and NAFLD

The non-synonymous PNPLA3 (rs738409) and TM6SF2 (rs58542926) single nucleotide polymorphisms (SNPs) are relatively common in the general population (minor allele frequencies vary with ethnicity but are approximately 20%–50% and 10%, respectively) (201). These variants, first linked with NAFLD by genome-wide association studies, are associated with an increased risk of steatohepatitis and more severe liver fibrosis (202-206). The genotype rs641738 at the MBOAT7-TMC4 locus is associated with increased hepatic fat content, more severe liver damage and increased risk of fibrosis (207) and genetic variants in GCKR rs780094 are also associated with an increased risk of NAFLD (208). *HSD17B13* has been reported to be relevant to NAFLD with several variants associated with decreased risk(209) In the largest GWAS on histologically characterised NAFLD (addressing the whole histological spectrum), a four-gene combination has been reported as a NAFLD risk modifier and of the above genes, included only PNPLA3, TM6SF2 and GCKR and

HSD17B13 (210). This study also ultimately confirmed *PNPLA3* as a risk factor for the full histological spectrum of NAFLD at genome-wide significance levels, therefore its consideration in diagnostic panels involving proteomic biomarkers was warranted to see if it improved diagnostic accuracies. Further candidates for NAFLD biomarkers have been suggested in recent paper exploiting hepatic transcriptome analysis in patients stratified by the presence of *PNPLA3* I148M variant. This study reported that at single gene level, Interleukin-32 (IL32) was the most strongly upregulated transcript (independent pf PNPLA3 variant carriage) in severe NAFLD where its serum circulating levels correlated with hepatic expression and were increased in patients with NAFLD thus proposing IL2 as a potential biomarker for the non-invasive assessment of NAFLD (strong correlation observed between hepatic mRNA and plasma IL32 levels, R=0.73, p=0.002(211)).

1.15.2. Epigenomics; DNA Methylation and liver fibrosis

Genetic variants have been characterised as having a strong association with particular liver disease aetiologies, however have limited diagnostic and prognostic capacities. Epigenetic modifications represent a collection of mechanisms that transform environmental insults into dynamic and heritable alterations of transcriptional potential and are gaining notoriety in both basic science and clinical studies. Epigenetic mechanisms reprogram hepatocytes allowing them to adapt to inflammation and oxidative stress caused by fat accumulation (212). To date, DNA methylation is the most extensively studied epigenetic modification, which involves the covalent addition of a methyl group to a cytosine yielding 5-methylcytosine (5mC).

Evidence for DNA Methylation and Liver Fibrosis: Application of a DNA methylation inhibitor (5-aza-2-deoxycytidine) to hepatic stellate cells (HSCs), blocks HSC proliferation and transdifferentiation to myofibroblasts demonstrating the fundamental role of DNA methylation in fibrogenesis (213). Methylome mapping has supported this observation and demonstrates DNA methylome remodelling in response to HSC transdifferentiation; with both quiescent and activated cells

displaying distinct 5-mC landscapes that involve changes across the entire genome with 'hot spots' of differential methylation at chromosomes 13, 19, 20, 21 and at the male Y chromosome (214). Significant methylome alterations are paralleled with changes in the expression of DNMTs and TETs which has led to functional studies implicating DNMT3a/b as a regulator of HSC transdifferentiation. Methylome re-configuration by methyl-CpG binding proteins (MBDs), which are responsible for both reading and interpreting the HSC methylome; play a key role in executing the phenotypic changes characteristic of fibrosis i.e. HSC transdifferentiation to the myofibroblast form. As an example, MeCP2 which is the most well characterised MBD, is induced in the earliest stages of HSC transdifferentiation and binds to methylated regions in the PPARγ gene promoter, resulting in transcriptional repression of PPARγ and loss of its inhibitory effects on the myofibroblast phenotype and collagen expression (215) (***figure 1.2)***. An exciting opportunity that has demonstrated its clinical potential is the relationship between alterations in the methylome of cell-free circulating DNA (ccfDNA) and liver fibrosis. The demonstration that remodelling of methylation at the PPARγ promoter in ccfDNA can be quantified and correlates with progression to fibrosis in chronic liver disease offers the opportunity for development of minimal-invasive liquid biomarkers in fibrotic chronic liver disease (168). This is an observation that will be validated in this study.

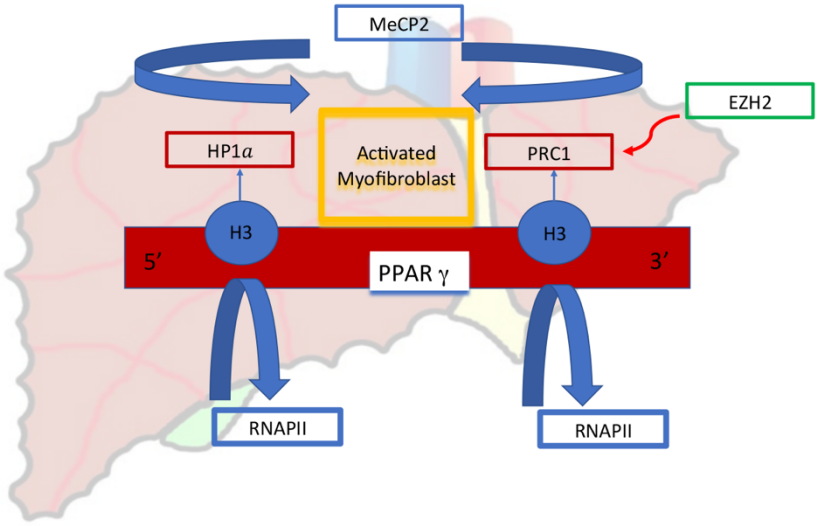


Figure 1.2: HSC transdifferentiation to myofibroblasts

Figure 1.2: HSC transdifferentiation to myofibroblasts involves PPARγ, silencing. Methylated CpGs within the PPARγ promoter recruit MeCP2, which then directs repressive H3K9me3-modifying enzymes to suppress transcription. In the downstream coding region of PPARγ, transcription elongation is suppressed by EZH2-mediated H3K27me3 modifications.(168)

1.15.3. Tissue specific methylation changes

Fibrotic specific epigenetic signatures (with a direct effect on liver function) have been derived from both liver tissue and circulating cell free DNA (167, 168).

1.15.4. Liver Biopsy derived methylation signatures

A recent study conducted in the Institute of Cellular Medicine (ICM) in a small cohort of well-characterised NASH showed that DNA methylation status of key fibrosis modifier genes loci (based on genomic DNA extracted from FFPE liver biopsy tissue) can stratify patients according to fibrosis severity (167). This supported the findings of an earlier study based on liver biopsy genomic DNA performed by Ana Mae Diel's group in Duke University, which examined differential DNA methylation in 69,247 CpG sites in liver biopsies from mild (F0-2) vs. advanced (F3-4) fibrosis patients. The majority of differentially methylated sites became hypomethylated with disease progression (76%), whereas 24% underwent hypermethylation (216)

1.15.5. Cell-free circulating DNA derived methylation signatures

Small fragments of DNA circulate in the peripheral circulation and are thought to originate from apoptotic cells and are thus representative of ongoing cell death (217) The concept of a liquid biopsy was coined in 1948 and ccfDNA was detected in human plasma approximately 3 decades ago (218). CcfDNA has been researched as a biomarker of NAFLD disease severity, however results have always been undermined by the lack of a definitive association between ccfDNA and NASH biology(168). Using a reference methylation atlas of 25 human tissues and cell types (219), it has been shown that plasma ccfDNA of healthy donors originates predominantly from white blood cells (55%), erythrocyte progenitor cells (30%), vascular endothelial cells (10%) and hepatocytes (1%). ccfDNA has had useful applications in medicine to date. Non-invasive pre-natal testing for foetal chromosomal abnormalities has been performed by analysing foetal DNA in maternal blood (220, 221); in organ transplant recipients, ccfDNA has been characterised for the early detection of graft rejection (222, 223) and mutated DNA in circulation of cancer patient has been characterised to diagnose and monitor disease (224). Unfortunately, considerable analytical and technical challenges associated with ccfDNA present challenges for biomarker development (218). This is in contrast to circulating miRNA signatures which have been proposed as adequate biomarkers for disease stage, global metabolic dysfunction and cardiovascular risk in NAFLD (169, 225-227).

1.15.6. Epigenetics in disease management

DNA methylation biomarkers in NAFLD considered to date have been classified as per the FDA BEST biomarker classification as having a ***diagnostic context of use***. However, changes in DNA methylation have been proposed as also potentially fulfilling the FDA 'dynamic' role, monitoring treatment response. Noteworthy examples include monitoring response to DMARDs in rheumatoid arthritis, steroids in paediatric asthma and response to cognitive behavioural therapy in paediatric anxiety disorders (228-230). Monitoring treatment response in NAFLD is also theoretically possible. For example, changes in the hepatic methylome was observed in obese patients who underwent bariatric surgery (231) and hepatic methylation

status of the mitochondrial enzyme MT-ND6 (which is associated with NAFLD severity) is modified by physical activity (232).

1.15.7. Benefits of a NAFLD 'Progression signature'

While disease diagnosis remains the crux of clinical practice, modern medicine now necessitates a framework incorporating patient prognosis and variability in disease outcome (233). Applied to the field of hepatology, while quantifying fibrosis stage remains essential for accurate diagnosis and staging; the development of an epigenetic progression signature would be extremely valuable in clinical practice to stratify disease management priorities. Epigenetic progression signatures have been successfully characterized in the field of oncology for example; methylation of p16INK4a and SFRP1 promoter hypermethylation is associated with poor prognosis in both colorectal and lung cancer and breast cancer respectively (234, 235). Epigenetic markers have started the laborious transition from bench to bedside where they are predicted to become integral in patient care provision. This study aims to characterize a methylation signature to prognosticate low and high risk disease in NAFLD, identifying those at risk of progressive fibrosis and those likely to remain stable.

1.16. Summary of objectives

(1) To establish the QoL and economic burden associated with NAFLD from a UK perspective. This will serve to complement available NAFLD morbidity and mortality data. This information will be gathered in a pre-anti fibrotic therapy NAFLD population and will provide foundation data to fully evaluate the impact of new anti-fibrotic treatments and possibly help direct future NAFLD health care policies

(2) To validate recently proposed structural fibrosis biomarkers in parallel to the performance of novel, proof of concept studies in the fields of epigenetics involving the characterisation of both diagnostic and prognostic biomarkers.

CHAPTER 2.

NAFLD DISEASE BURDEN: EXPLORING EMOTIONAL, MENTAL, PHYSICAL AND SOCIAL FUNCTIONING IN NAFLD: DATA FROM THE EUROPEAN NAFLD REGISTRY; AN ANALYSIS OF PATIENT REPORTED OUTCOMES (PROS) IN NAFLD

2.1. INTRODUCTION

In Europe, 30% of the population are estimated to have NAFLD (16, 236, 237). A common misconception is that patients suffering from chronic liver disease, especially in its formative years are asymptomatic (238). The burden of disease in NAFLD is complicated by "unspecific symptoms" reported to negatively impact patient quality of life (QoL) (103). QoL data is becoming valuable in both scientific and clinical fields in the development of NAFLD specific treatments (239). However, a recent global literature review evaluating QoL studies in NAFLD identified only 20 high quality full text articles, of which, only 8 articles were appropriate for data extraction reflecting the paucity of high quality published studies describing NAFLD QoL impairment from the patient perspective (240).

2.1.1. Impaired HRQL in chronic liver disease

Review of the literature consistently demonstrates that chronic liver disease patients report poorer HRQL metrics in both cirrhotic and non-cirrhotic populations compared to both healthy controls and normative data (241, 242). Exploring further, the degree of HRQL impairment that exists between liver diseases is conflicting. Fortunately, most researchers involved in QoL studies in liver disease utilise either the validated generic SF-36 or the disease specific CLDQ, with highly correlated respective sub-domain scores enabling valid comparisons (243). In a study by Dan et al, more burdensome disease has been reported in NAFLD compared to HBV, ALD and cholestatic liver disease patients and in the majority of cases involving HCV (100). A further US study found that patients with NAFLD had significantly lower SF-36 scores than patients with ALD, chronic viral hepatitis or cholestatic liver disease (101). However, an additional two studies from the US and UK found no difference in QoL scores between disease aetiologies (244, 245). As a result, no consistent evidence based pattern can be derived, perhaps due to heterogeneity in study design (246). Isolating NAFLD liver disease, a recent review of relevant QoL literature has concluded that NASH affects QoL, in particular physical functioning and fatigue (240). NASH patients have been found to have a lower QoL versus normative and NAFL populations but not versus other chronic liver disease (240). In a recent study, Younossi et al compared HRQL scores in patients with chronic liver disease (CLD) (n=1338) to those with NASH (n=1338) using 3 PROs (CLDQ, Work productivity and activity impairment and Short Form-36). Patients with NASH

and advanced fibrosis have more impairment of their physical health–related scores than patients with CHC with advanced fibrosis after adjustment for demographic parameters, cirrhosis, and history of psychiatric disorders. Patients with NASH had significantly lower HRQL scores related to physical health and Fatigue of CLDQ ($P < 0.02$) (247).

2.1.2. Fatigue and NAFLD

Evidence to date, from both patient descriptions in clinic and qualitative research in focus groups has shown that fatigue is one of the most prevalent symptoms reported in patients with chronic liver disease (248, 249). In this study, patient experience of fatigue will be captured using the fatigue impact score (FIS) and functional impact assessed by means of the Epworth sleepiness scale (ESS). Preliminary review of the literature has shown a strong positive correlation between BMI and NAFLD, supporting the "tired fat people" phenotype (248). However, in a study by Newton et al, this concept has been wholly challenged. The UK group demonstrated that patients with NAFLD have elevated levels of fatigue and reduced physical activity levels independent of age, sex, BMI or the presence of depression (248). This would suggest that fatigue in NAFLD has a unique pathogenesis, with a pathway that warrants elucidation and if found, development of targeted treatment.

2.1.3. Mental Health and NAFLD

Prevalence of depression and the lifetime incidence of major depressive disorders are higher in patients with NAFLD than with other chronic liver diseases (250, 251). Possible hypotheses stem from the strong association between NAFLD and insulin resistance (252-254). There is also evidence to suggest that mood disorders are associated with liver disease severity. Hepatocyte ballooning has been shown to correlate with depression in a dose dependent manner (255). There is also a small study in NAFLD patients which demonstrates a correlation between steatosis grade and the diagnosis of a major depressive disorder (251). However, both "NASH" and steatosis grade are dynamic states and the studies discussed report "current" depressive symptoms (as captured by PRO scales) and histological features of NASH as recorded on liver biopsy which were not always correlated in real time. Observations are also criticized as no intervention arm existed to examine the potential influence of psychotropic drugs on liver histology. As a result, it is difficult to

derive a robust conclusion and to date potential mechanisms have not been elucidated. However, fibrosis state is not subject to such dynamic fluctuations and in NAFLD patients the severity of subclinical depression has been found to correlate with fibrosis stage (256). Looking to in-vivo models to derive a mechanism, it is postulated that serotonin may modulate the ductular reaction and fibrogenic repair responses in portal areas (257). MRI studies have also shown that as patients progress to more advanced fibrosis/cirrhosis, progressive cerebral atrophy is observed, which persists after liver transplantation and is speculated to contribute to overall reduced QoL scores observed in CLD patient cohorts (258).

2.1.4. Significance of liver histology and metabolic co-morbidities in QoL in NAFLD

There is a conflicting body of literature in NAFLD regarding the influence of histological disease severity on total symptom burden (248, 259). In chronic liver disease, there has been evidence to suggest that inflammation plays a dominant role on HRQL. Improvements in HRQL have been reported in NASH patients with F2/F3 fibrosis where an improvement in fibrosis by one stage resulted in improved HRQL measures (260). The divergence of fibrosis on mortality and QoL potentially reflects unique underlying pathogenic mechanisms that contribute to disease progression and loss of QoL in NAFLD. It has also been shown that the number of co-morbidities (largely metabolic syndrome related) and medications negatively correlate with HRQL in patients with chronic liver disease (261). Impaired QoL contributes significantly to patient disease burden in NAFLD, therefore with the formulatory of anti-fibrotic and NASH targeted therapies pending FDA approval a cost-benefit analysis cognisant of QoL data will be valuable.

2.1.5. Assessments of PROs in NAFLD

Currently, Health related quality of life (HRQL) is defined as the “subjective assessment of the impact of disease across the physical, psychological, social and somatic domains of functioning and well-being” (262). Both objective and subjective assessments of HRQL can be employed (246), the former being more amenable to analysis while the latter measurements are a truer assessment of the patient experience (246). HRQL scales can be broadly categorised into generic and disease specific measurements. Generic scales are a

metric of physical, mental and social aspects of a health status but there is a lack of sensitivity to clinically important changes e.g. Short Form-36 (SF-36) questionnaire. Various tools have been adapted and are available to specifically address symptoms in chronic liver disease (263). A disease specific questionnaire (Chronic Liver Disease Questionnaire) was employed in this thesis and allowed the evaluation of how disease severity impacts on quality of life (QoL). These measures were supplemented with domain specific scales to focus on specific areas of interest (Depression, fatigue and daytime sleepiness) balancing the benefit of additional information against participant burden (264). However, the CLDQ has been criticised for having shown significant differences relating to gender and may have a higher sensitivity in women compared to men. Furthermore, analysis of CLDQ performance in NAFLD has shown that the ability of the standard CLDQ to differentiate at a more in-depth level was questionable as NAFLD patients consistently scored within a range of 2.5 points on the 7 point Likert scales. Younossi et al recently developed the CLDQ-NAFLD for efficacy trials in NAFLD. The domains of CLDQ-NASH showed good to excellent internal consistency (Cronbach's α values were 0.80 to 0.94). Known-group validity tests indicated that the instrument consistently discriminated between patients with NASH based on the presence of cirrhosis, obesity, psychiatric comorbidities, fatigue and type 2 diabetes (265).However, the CLDQ score was adopted in this study this was part of a collaboration with other study sites which had previously used the CLDQ.

2.1.6. Study aims and Objectives

This study aimed to perform a comprehensive assessment of QoL burden of NASH from the patient perspective in a large, well characterised cohort of patients with histologically proven NAFLD.

- The assessment will utilise disease specific scales and domain specific questionnaires.
- This study will employ a systemic approach to first explore the impact of NAFLD on patient's general well-being and then proceed to classify research findings into thematic categories (fatigue and depression) using domain specific assessments to explore areas of scientific vagueness and create a foundation for future research.

2.2. MATERIALS AND METHODS

2.2.1. Study Design and Participants

Flow of patients through the study is shown in ***Figure 2.1*** Patients were recruited at the Freeman Hospital Liver Unit, Newcastle Upon Tyne Hospitals, NHS Trust as part of the European NAFLD Registry. The UK arm of the NAFLD registry is a continuously updated cohort of consecutive patients attending the Tertiary Liver Clinic who had NAFLD diagnosed histologically.

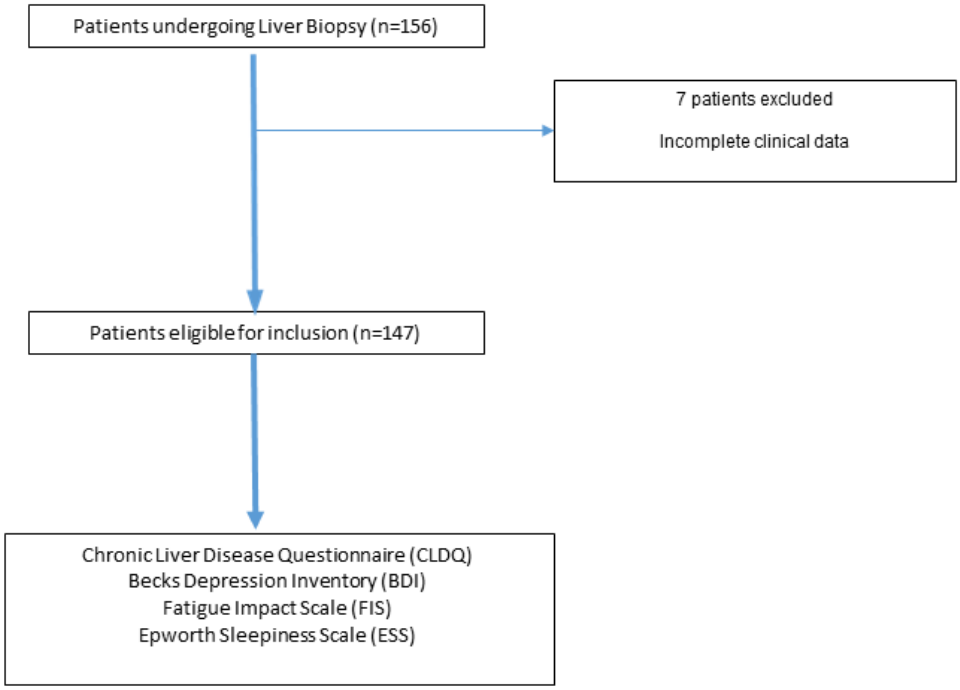


Figure 2.1. Study Design

2.2.2. Ethics

This study received approval from the Ethics Committee under the remit of EPoS (Elucidating pathways of steatohepatitis).

2.2.3. Clinical and laboratory assessments

Gender, age and body mass index (BMI; weight (kg)/height (m^2)) OF NAFLD patients were recorded for all patients at time of index liver biopsy. Patients were classified as having type 2 diabetes (T2DM) if HbA1c >6.5% or they were receiving dietary, oral hypoglycaemic drug or insulin treatment for T2DM. Blood tests taken at the time of liver biopsy were used to calculate the simple non-invasive scores. (Haematology, Liver profile (ALT inclusive), AST). Laboratory results were obtained at time of attendance, within a maximum of 30 days of liver biopsy.

2.2.4. Liver Biopsy Procedure

Liver biopsies were performed at each centre as per unit protocol. Target biopsy length was ≥15mm. Biopsies were stained with haematoxylin and eosin and Masson's trichrome. Histological diagnosis, grade of steatosis and scoring for NAFLD activity and fibrosis stage were performed by expert liver pathologists at each study site (45).

2.2.5. Histological Assessment

Brunt et al described a semi-quantitative evaluation of the lesions associated with NASH. His system introduced the concepts of 'grading' activity and 'staging' fibrosis in NAFLD, based on the premise that disease activity assessment could be more accurately summarised as a combination of steatosis, inflammation and ballooning determining the grade (48). The National Institute of Diabetes and Digestive and Kidney Diseases-sponsored NASH Clinical Research Network (CRN) proposed and validated an updated scoring system to aid clinical trials of the CRN, and to encompass the whole spectrum of NAFLD. (45) The NAFLD activity score (NAS) was graded from 0 to 8 including scores for steatosis (0–3), lobular inflammation (0-3) and hepatocellular ballooning (0-2). Fibrosis was staged from 0 to 4. 'NASH' was defined as steatosis with hepatocyte ballooning degeneration and inflammation +/– fibrosis (266). NASH was defined as NAS score $\geq$ 4. Staging of fibrosis follows a five-tier method (0-4) indicating progression of fibrosis from zone 3 peri-sinusoidal, to portal, to bridging to cirrhosis. (48) 'NAFL' was defined as steatosis only, or steatosis with mild inflammation without hepatocyte ballooning degeneration. (267).

2.2.6. Patient recruitment criteria (NAFLD Diagnosis)

Inclusion criteria: Patients eligible for inclusion were ≥18 years, with suspected NAFLD undergoing a diagnostic/staging liver biopsy on clinical grounds.

Exclusion criteria: Patients were excluded if they had evidence of coexistent liver disease; if they were reviewed for alcoholic cirrhosis; alcohol dependency; if they consumed greater than 30g of alcohol per day for males or greater than 20g per day for females.; hepatic viral disease; autoimmune liver disease; hepatic tumors; suspicion of drug induced liver injury; miscellaneous liver pathologies e.g. acute hepatitis, vascular etiologies or cholangiopathies.

2.2.7. Assessment Tools

A series of questionnaires, validated for self-completion were employed to quantify (1) general QoL impairment and (2) domain specific impairment. All assessment tools have been previously used in subjects with chronic liver disease. Patients completed questionnaires during regular out-patient visits and within 6 months of the diagnostic liver biopsy.

2.2.7.1. Chronic Liver Disease Questionnaire (CLDQ)

Quality of life (QoL) was quantified with the liver specific instrument the Chronic Liver Disease Questionnaire (CLDQ). It consists of 29 items which are quantified on a 7-point Likert scale (range 1-7) representing the frequency of CLD associated symptoms over the preceding two weeks. It consists of 6 sub-scale scores covering abdominal symptoms, fatigue, systemic symptoms, emotional problems and worry associated with CLD. Each overall domain score is divided by the number of domain components so that CLDQ components can be presented on a 1-7 scale with 1 indicating the worst and 7 the best QoL (263, 268). CLDQ scores for populations with PBC, HCV and HBV were obtained from the Chronic Liver Disease Database in Fairfax hospital, Virginia, USA. Normative data, published elsewhere (USA) was also available for comparative analysis (269).

2.2.7.2. Beck Inventory Version 2

Depressive symptoms were quantified using the Beck Inventory Version 2 (BIV2). It consists of 21 items quantified on a 4-point scale from 0 (symptom absent) to 3 (severe symptoms), measuring affective, cognitive, somatic and vegetative symptoms in line with the DSM-IV criteria for major depression. The highest score assigned for each of the 21 items is added to achieve the total score (the minimum score is 0, the maximum score is 63). Higher scores indicate greater symptom severity. In non-clinical populations, scores above 20 indicate moderate or severe depression, while scores less than 20 indicate mild or no depression (270)

2.2.7.3. Fatigue Impact Scale

The Fatigue Impact scale was used to quantify fatigue. It consists of 40 items quantified on a 5-point scale from 0 (no problem) to 4 (extreme problem), providing a continuous scale of 0-160. It consists of 3 sub-scale scores (each containing 10 items) measuring the impact of fatigue on cognitive, physical and psychosocial functioning with higher scores reflecting increasing fatigue. Scores $\geq$ 29 indicate significant fatigue. External validity has been established in a wide spectrum of chronic diseases including liver disease (271, 272)

2.2.7.4. Epworth Sleepiness Scale

The Epworth Sleepiness Scale was used to assess daytime hypersomnolence. The questionnaire consists of 8 different activities, differing in their somnificity and respondents are required to rate on a 4-point scale (0-3) their chances of having fallen asleep while engaged in these activities. The total ESS score provides an estimate of a general characteristic termed the person's 'average sleep propensity' (ASP). A score $\geq$ 10 is indicative of significant daytime somnolence (273).

2.2.8. Statistical Analysis

Descriptive statistics were calculated for all variables (means and standard deviations for continuous variables and frequencies and percentages for categorical variables). Analysis of covariance (ANCOVA) was used to test the main and interaction effects of categorical variables (histological disease stage) on a continuous dependent variable (PRO scales),

controlling for the effects of selected other continuous variables, which co-vary with the dependent. Associations between two variables were assessed using univariate regression analysis. The Mann-Whitney-U-Rank test or Chi-square test was used to calculate differences between two groups while the Kruskal-Wallis rank test was used to compare multiple different groups. This was followed by Dunn's multiple comparison test where appropriate. Correlations between individual parameters were assessed by Spearmann rank test. All tests were two-tailed and considered statistically significant when $p<0.005$. A one sample T-test was performed to see if there was a statistically significant difference in mean CLDQ scores in the NAFLD population compared with normative data for healthy controls and other liver disease aetiologies published elsewhere (269) . All statistical analyses were performed using SPSS software version 24.0 (SPSS Inc, Chicago, USA).

2.3. RESULTS

2.3.1. Patient Characteristics

The clinic-demographic characteristics of study population are shown in **Table 2.1** The cohort consisted of 147 subjects. The mean age was 53 +/- 13 years. 58% were male. 91% were obese (mean BMI was 35 kg/m^2). Metabolic syndrome components hypertension, hyperlipidaemia and T2DM were present in 57%, 57% and 61% of the study population respectively. NASH (NAS≥4) was detected in 42% of the cohort (n=62). 58% of the population had mild fibrosis (F0-F2) while 42% had advanced fibrosis (F3-F4). The mean CLDQ score is 4.72, the mean FIS is 79.45 (significant fatigue if score ≥29), the average ESS is 7.63 (hypersomnolence ≥10) and the average BIV2 score is 13.75 (moderate/severe depression>20). 100% of the cohort had significant fatigue, 22% had significant hypersomnolence (n=33) and 23% (n=34) had moderate to severe depression.

Table 2.1. Clinico-demographic characteristics	
Variable	**Study Cohort (n=147)**
Patient demographics and metabolic profile	
Gender (Male)	85 (58%)
Age	53 +/- 13
BMI	35 +/- 5
Obesity	134 (91%)
T2DM	90 (61%)
Hypertension	87 (57%)
Hyperlipidaemia	88 (57%)
ALT	93 +/- 62
AST	60 +/-33
Albumin	44 +/-3
Platelet count	249 +/-79
Cholesterol	5.3 +/-1.3
Triglyceride	2.6 +/- 1.8
Histology	
NASH (NAS score>4)	62 (42%)
Steatosis (0/1/2/3)	1/33/79/34
Ballooning (0/1/2)	44/72/31
Lobular inflammation (0/1/2/3)	27/69/49/2
Fibrosis (0/1/2/3/4)	29/29/28/40/21
Patient Reported Outcomes	
CLDQ	4.72 +/- 1.31
FIS	79.45 +/- 33.89
ESS	7.63 +/- 4.84
BIV2	13.75+/- 10.95
Data are expressed as *number (percentage) or #mean (standard deviation). BMI= body mass index; obesity defined as BMI>30kg/m^2; ALT = Alanine aminotransferase; AST= aspartate aminotransferase	

2.3.2. Preliminary Exploration of the influence of NAFLD histology on CLDQ, FIS, ESS and BIV2 Scores

To determine whether patient reported outcomes (PROs) were influenced by liver disease severity, a one-way ANCOVA was conducted for each scale and liver histology parameter. Likely confounding factors were included as covariates in each analysis *(Age, Gender, BMI, T2DM, ALT, AST, Albumin, Platelets).*

Chronic Liver Disease Questionnaire. No significant difference in CLDQ scores were reported for steatosis, ballooning or fibrosis. For lobular inflammation, there was a significant difference in CLDQ (F= 3.802, p= 0.012). However, the effect size was negligible at 0.082. CLDQ scores were highest in those with grade 1 Lobular inflammation (5.02 +/- 1.3).

FIS scores. No significant difference in FIS scores were observed for steatosis, ballooning or fibrosis. For lobular inflammation grade, there was a significant difference in FIS (F= 4.908, p= 0.003). However, the effect size was negligible at 0.105. FIS scores were highest in those with grade 3 Lobular inflammation (80 +/- 33).

ESS Scores No significant difference in ESS scores were reported for steatosis, ballooning or lobular inflammation. For fibrosis, there was a significant difference in ESS (F=2.470, p=0.049). However, the effect size was negligible at 0.082. ESS scores were highest in those with stage 2 fibrosis (9.48 +/- 4.5).

Beck Depression Inventory Version 2. Steatosis, ballooning, lobular inflammation or fibrosis did not influence BIV2 scores. **(Table 2.2)**

Table 2.2 Impact of liver histological parameter stage on PRO scales		
F	**P-Value**	**Eta Squared- Effect Size**
Chronic Liver Disease Questionnaire		
Steatosis		
2.474	0.065	0.055
Ballooning		
1.016	0.365	0.016
Lobular inflammation		
3.802	**0.012**	0.082
Fibrosis		
0.969	0.427	0.030
Fatigue Impact Score		
Steatosis		
0.657	0.580	0.015
Ballooning		
2.202	0.115	0.034
Lobular inflammation		
4.908	**0.003**	0.105
Fibrosis		
1.665	0.162	0.051
Epworth Sleepiness Score		
Steatosis		
0.822	0.484	0.022
Ballooning		
2.239	0.111	0.038
Lobular inflammation		
0.976	0.407	0.026
Fibrosis		
2.470	**0.049**	0.082
Beck's Depression Inventory Version 2		
Steatosis		
0.539	0.657	0.013
Ballooning		
0.499	0.608	0.008
Lobular inflammation		
1.996	0.118	0.045
Fibrosis		
0.490	0.743	0.015
*Statistical test- ANCOVA; **Covariates: Age, Gender, BMI, T2DM, ALT, AST, Albumin, Platelets		

2.3.3. Preliminary exploration of CLDQ scores

Mean CLDQ score was 4.73 +/- 1.3. The lowest scores were reported in the subcategories "systemic symptoms" with a value of 3.94 +/- 1.1, followed by fatigue with a value of 4.12 +/- 1.6. "Abdominal symptoms" and "Activity" had the highest scores. Scores in the NAFLD population were significantly lower than the healthy control population and the HBV population. No differences were observed in the subdomains "fatigue" and "emotional functioning" between HCV and NAFLD and no differences in "emotional functioning" was observed between NAFLD and PBC. (**Table 2.3)**

Table 2.3 Health-related quality of life scores compared to normative data and other CLD

CLDQ N=147	**NAFLD Population**	****Normative Data**	***P-Value**	****PBC**	**P-Value**	****HCV**	**P-Value**	****HBV**	**P-Value**
CLDQ Total	4.73 +/- 1.3	6.0+/- 1	<0.0001	4.4+/- 1.3	0.003	4.4+/- 1.6	0.003	6.0+/- 0.9	<0.0001
Abdominal symptoms	5.24 +/- 1.6	6.0+/- 1	<0.0001	4.6+/- 1.8	<0.0001	5.0+/- 1.9	0.075	6.2+/- 1.0	<0.0001
Fatigue	4.12 +/- 1.6	5.0 +/-1	<0.0001	3.7+/- 1.8	0.002	4.0 +/- 1.9	**0.355**	5.6 +/- 1.0	<0.0001
Systemic symptoms	3.94 +/-1.1	6.0+/- 1	<0.0001	4.7 +/- 1.3	<0.0001	4.9+/- 1.4	<0.0001	6.2+/- 1.0	<0.0001
Activity	5.2 +/- 1.5	6.0+/- 1	<0.0001	4.9+/- 1.6	0.011	4.7+/- 1.8	<0.0001	6.3+/- 1.1	<0.0001
Emotional functioning	4.6 +/- 1.6	6.0+/- 1	<0.0001	4.7+/- 1.2	**0.295**	4.6+/- 1.4	**0.759**	5.8+/- 1.0	<0.0001
Worry	4.91 +/- 1.6	7.0 +/- 1	<0.0001	4.4+/- 1.7	<0.0001	4.4+/- 1.8	<0.0001	5.9+/- 1.1	<0.0001

Reported means +/- st devs; *Statistical test; one Sample T Test ;**Data obtained from other publications (269)

The correlation of total CLDQ score with clinico-demographic patient details and liver histology is shown in **Table 2.4**. There was a significant negative linear correlation between total CLDQ scores and male gender, BMI, NASH and the presence of lobular inflammation (LI) on liver biopsy.

Table 2.4. Correlation of CLDQ scores with patient clinico-demographic details		
Variable	**Correlation with Total CLDQ scores**	**P-value**
Gender (Male)**	-0.298	**<0.0001**
Age	0.083	0.319
BMI	-0.307	**<0.0001**
T2DM	-0.125	0.130
ALT	0.001	0.989
ALT>40	-0.154	0.063
AST	-0.101	0.234
Albumin	0.112	0.175
Platelet count	-0.101	0.223
Histology		
NASH (NAS Score>4)	-0.172	0.038
Steatosis (1/2/3)	-0. 114	0. 171
Ballooning (0/1/2)	-0.109	0.188
Lobular inflammation (0/1/2/3)	-0.200	**0.015**
Fibrosis (0/1/2/3/4)	-0.086	0.303
BMI= body mass index; obesity defined as BMI>30kg/m^2; ALT = Alanine aminotransferase; AST= aspartate aminotransferase Histological findings were scored according to the criteria proposed by Kleiner et al.(45) Statistical test; Spearman's correlation coefficient (Rs)		

2.3.3.1. Patient demographics; Age, gender and CLDQ scores

A weak, significant negative linear correlation existed between total CLDQ scores and gender (Rs= -0.3, p= <0.0001). Women had a significantly lower total CLDQ score than men (4.27 versus 5.1, p= <0.0001). All 6 CLDQ subcategories were significantly lower in females. No significant correlation existed between total CLDQ and age (p=0.083). There was a trend toward lower scores in all sub-categories excluding fatigue with increasing age, however these differences were not statistically significant. **(Table 2.5)**

Table 2.5 Age, gender and CLDQ scores							
CLDQ	**Patient characteristics - Age and Gender**						
	Total (n=147)	**Male (n=85)**	**Female (n= 62)**	**P-value**	**Age >70 (n=136)**	**Age<70 (n=11)**	**P-value**
Total score	4.73 +/- 1.3	5.1 +/- 1.2	4.27+/- 1.3	<0.0001	4.72 +/- 1.3	4.84 +/- 1.2	0.857
Abdominal symptoms	5.24 +/- 1.6	5.62+/- 1.4	4.71 +/- 1.7	0.001	5.22 +/- 1.6	5.48 +/- 1.8	0.435
Fatigue	4.12 +/- 1.6	4.37 +/- 1.6	3.78 +/- 1.6	0.029	4.15 +/- 1.6	3.71 +/- 2	0.409
Systemic symptoms	3.94 +/- 1.1	4.17 +/- 1.1	3.62 +/- 1.1	0.003	3.92 +/- 1.2	4.2 +/- 1.0	0.540
Activity	5.2 +/- 1.5	5.51 +/- 1.4	4.82 +/- 1.6	0.004	5.21 +/- 1.5	5.3 +/- 1.8	0.894
Emotional functioning	4.6 +/- 1.6	4.94+/- 1.5	4.04 +/- 1.7	0.001	4.52 +/- 1.6	5.0 +/- 1.4	0.322
Worry	4.91 +/- 1.6	5.3 +/- 1.4	4.43 +/- 1.8	0.005	4.92 +/- 1.7	4.8 +/- 1.6	0.768
*Data are expressed as means and standard deviations; statistical test; Mann-Whitney U test							

2.3.3.2. Patient metabolic profile and CLDQ scores

A moderate negative linear correlation existed between the presence of obesity and total CLDQ scores (Rs=-0.307, p=<0.0001). Total CLDQ scores were significantly lower in obese versus normal and overweight patients (4.66 versus 5.44, p= 0.036). Scores were also significantly lower in the subcategories “fatigue”, “systemic symptoms” and “activity”**.** No significant correlation existed between the presence of diabetes and the total CLDQ score (Rs= -0.125, p=0.130). There was a trend toward higher scores in the non-diabetic subset, only reaching statistical significance in the sub-categories “fatigue”, “activity” and “worry”. **(Table 2.6)**

Table 2.6. Obesity, diabetes and CLDQ scores							
CLDQ	**Metabolic Syndrome Factors**						
	Total (n=147)	**BMI<30 (n=13)**	**BMI>30 (n=134)**	**P-value**	**T2DM (n=90)**	**No T2DM (n=57)**	**p-value**
Total score	4.73 +/- 1.3	5.44 +/- 1.1	4.66 +/- 1.3	**0.036**	4.61 +/- 1.3	4.92 +/- 1.4	0.130
Abdominal symptoms	5.24 +/- 1.6	5.67 +/- 1.2	5.20 +/- 1.6	0.432	5.15 +/- 1.6	5.38 +/- 1.6	0.343
Fatigue	4.12 +/- 1.6	5.0 +/- 1.4	4.0 +/- 1.6	**0.033**	3.90 +/- 1.5	4.47 +/- 1.7	**0.044**
Systemic symptoms	3.94 +/- 1.1	4.57 +/- 1.1	3.89 +/- 1.1	**0.023**	3.83 +/- 1.1	4.11 +/- 1.2	0.081
Activity	5.2 +/- 1.5	6.08 +/- 1	5.13 +/- 1.5	**0.026**	4.99 +/- 1.5	5.57 +/- 1.4	**0.014**
Emotional functioning	4.6 +/- 1.6	5.21 +/- 1.5	4.50 +/- 1.6	0.126	4.58 +/- 1.6	4.53 +/- 1.7	0.905
Worry	4.91 +/- 1.6	5.68 +/- 1.2	4.84 +/- 1.7	0.089	4.74 +/- 1.6	5.18 +/- 1.7	**0.046**
*Data are expressed as means and standard deviations; statistical test; Mann-Whitney U test							

2.3.3.3. NAFLD disease severity and CLDQ scores

A total of 21 patients had cirrhosis (F4). No significant correlation exists between total CLDQ and the presence of cirrhosis (Rs=-0.073, p=0.382). There is a non-significant trend towards higher CLDQ scores in non-cirrhotic NAFLD patients. 86 patients had mild or intermediate fibrosis (F0-F2). No significant correlation exists between total CLDQ score and mild disease (Rs=0.119, p=0.151). There is a trend towards higher CLDQ scores in patients with mild disease which was significant in the subcategories “fatigue”, “activity” and “worry”. 62 patients had NASH (NAS≥4). A weak negative correlation exists between total CLDQ and the presence of NASH (Rs=-0.172, p=0.038). CLDQ scores are higher in patients with NAFL which reaches statistical significance in the subcategories “abdominal symptoms”, “fatigue”, “systemic symptoms” and “activity”. **(Table 2.7).**

Table 2.7. CLDQ scores and NAFLD histology										
CLDQ	**NAFLD Disease Severity**									
	Total (n=147)	**F0123** (n=126)	**Cirrhosis (n=21)**	**P-value**	**Mild F012 (n=86)**	**Severe F34 (n=61)**	**p-value**	**NASH NAS≥4 (n=62)**	**NAFL (n=85)**	**P-Value**
Total score	4.73 +/- 1.3	4.76 +/- 1.3	4.53 +/- 1.1	0.380	4.85 +/- 1.4	4.56 +/- 1.24	0.150	4.49 +/- 1.2	4.90 +/- 1.3	**0.038**
Abdominal symptoms	5.24 +/- 1.6	5.29 +/- 1.6	4.94 +/- 1.7	0.294	5.27 +/- 1.6	5.19 +/- 1.7	0.799	5.10 +/- 1.5	5.38 +/- 1.7	0.259
Fatigue	4.12 +/- 1.6	4.17 +/- 1.6	3.84 +/- 1.3	0.316	4.33 +/- 1.6	3.82 +/- 1.6	**0.044**	3.73+/- 1.6	4.41 +/- 1.5	**0.010**
Systemic symptoms	3.94 +/-1.1	3.98 +/- 1.2	3.70 +/- 1	0.207	4.10+/- 1.2	3.77 +/- 1.1	0.064	3.70 +/- 1.1	4.16 +/- 1.1	**0.018**
Activity	5.2 +/- 1.5	5.20 +/- 1.5	5.3 +/- 1.3	0.811	5.44 +/- 1.4	4.91 +/- 1.5	**0.019**	4.87+/- 1.3	5.47 +/- 1.6	**0.004**
Emotional functioning	4.6 +/- 1.6	4.56 +/- 1.6	4.55 +/- 1.6	0.883	4.53 +/- 1.7	4.59 +/- 1.6	0.894	4.42 +/- 1.6	4.66 +/- 1.7	0.342
Worry	4.91 +/- 1.6	4.97 +/- 1.6	4.56 +/- 1.7	0.232	5.13 +/- 1.7	4.61 +/- 1.5	**0.015**	4.68 +/- 1.6	5.10 +/- 1.7	0.062
*Data are expressed as means and standard deviations; statistical test; Mann-Whitney U test										

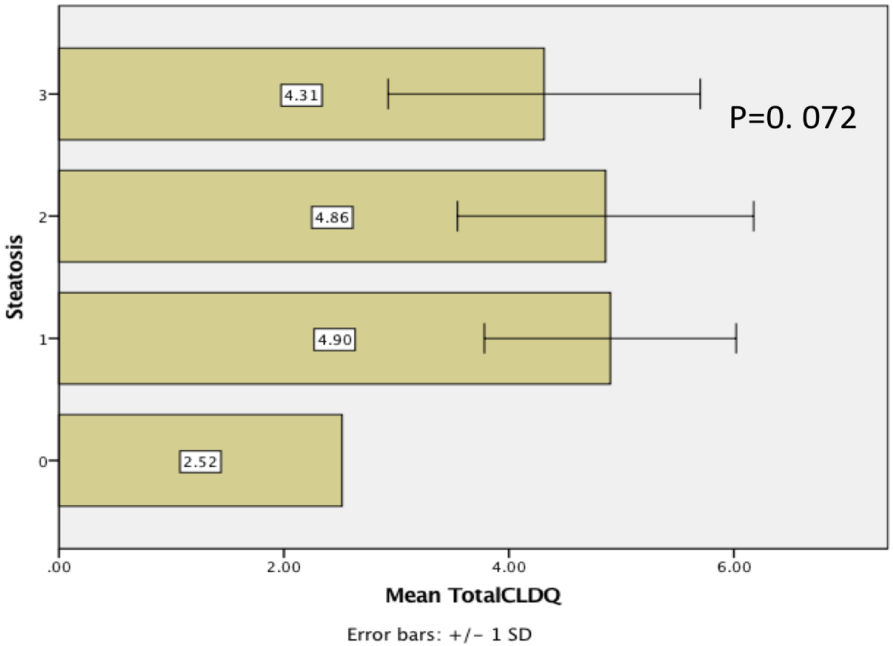
Specific histological parameter grades in NAFLD have a variable impact on CLDQ scores **(Table 2.8, Figure 2.2)**. Patients with more severe hepatic steatosis, ballooning and fibrosis had a trend towards lower scores however these trends did not reach statistical significance. More severe lobular inflammation (grade 3 vs grade 1: 3.03 vs 5.00; p = 0.046 and grade 2 vs grade 1:4.38 vs 5.00; p=0.017) were associated with lower CLDQ scores. This is in keeping with the earlier ANCOVA analysis **(Table 2.2),** when patient demographic, metabolic and biochemical characteristics were controlled for (F= 3.802, p= 0.012). CLDQ scores were highest in those with grade 1 Lobular inflammation (5.02 +/- 1.3). Patients with a more severe grade of LI had a significant trend to lower scores in the subcategories "fatigue", "systemic symptoms", "activity" and "worry".

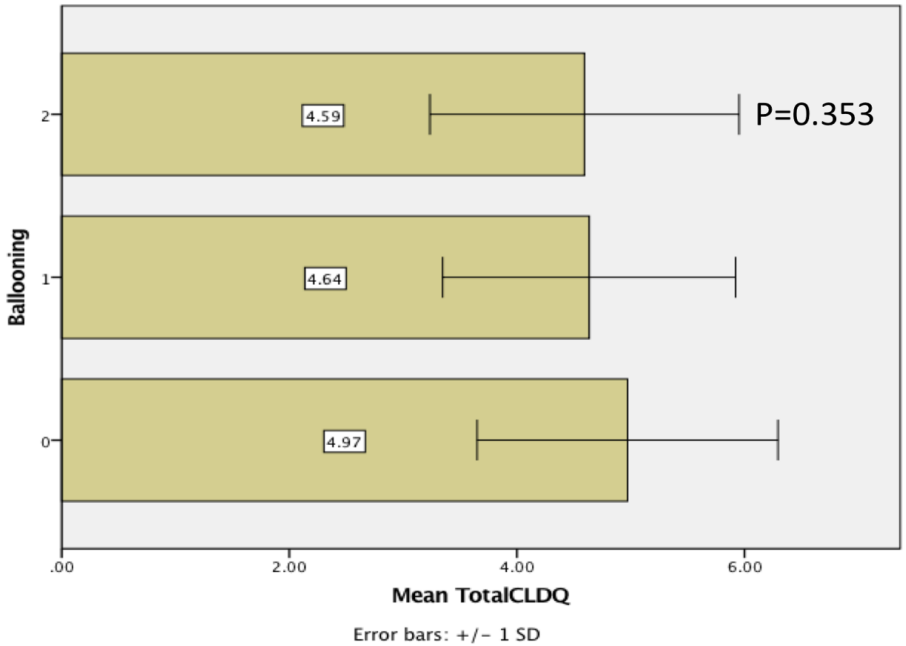
Table 2.8. CLDQ subdomain correlations with NAFLD histology

	Total CLDQ Score	P-value	Abdominal	P-value	Fatigue	P-value	Systemic	P-value	Activity	P-value	Emotional	P-value	Worry	P-value
Steatosis	-0.114	0.171	-0.130	0.116	-0.110	0.185	-0.135	0.103	-0.143	0.083	-0.068	0.413	-0.098	0.235
Ballooning	-0.109	0.188	-0.64	0.440	-0.183	**0.026**	-0.177	**0.032**	-0.163	**0.049**	-0.007	0.934	-0.078	0.349
Lobular Inflammation	-0.200	**0.015**	-0.100	0.229	-0.275	**0.001**	-0.180	**0.029**	-0.247	**0.003**	-0.080	0.333	-0.192	**0.020**
Fibrosis	-0.086	0.303	-0.032	0.704	-0.155	0.061	-0.125	0.130	-0.133	0.109	-0.040	0.635	-0.144	0.081

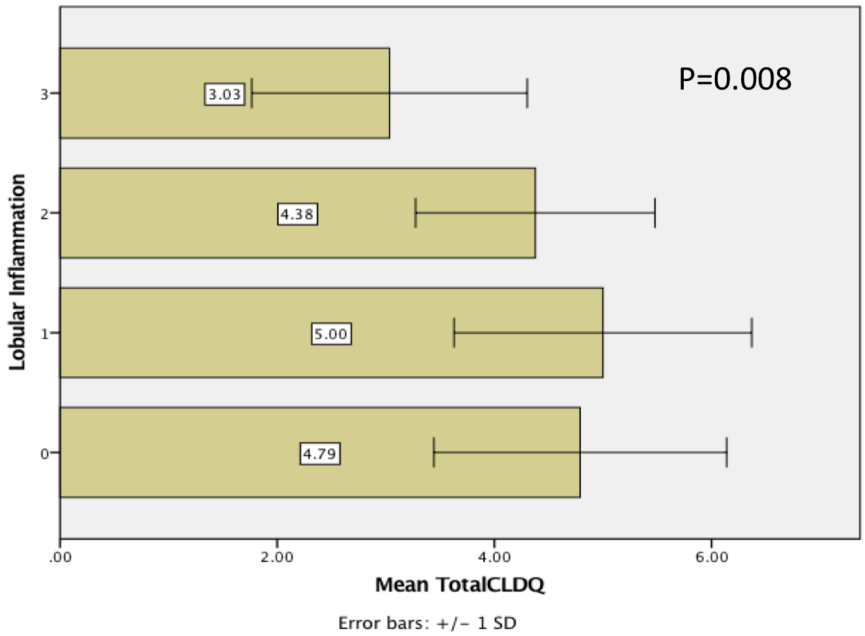
*Statistical test; Spearmans correlation co-efficient (Rs)

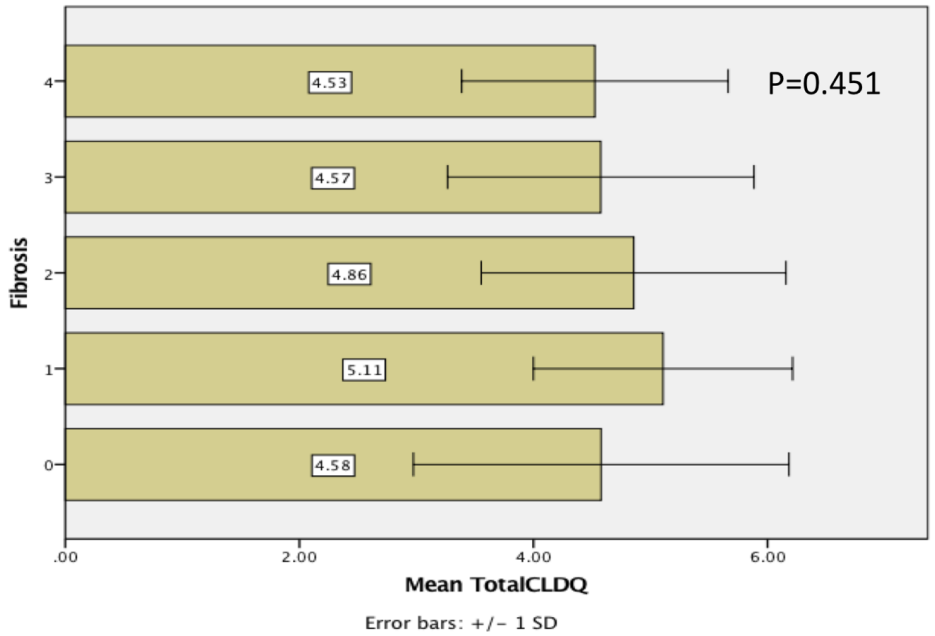
Figure 2.2. CLDQ Scores and NAFLD histology





Steatosis	Mean CLDQ score	N (%)	Ballooning	Mean Value	N (%)
0	4.31	1 (0.7%)	0	4.97	44 (29.9%)
1	4.3	33 (22.4%)	1	4.54	72 (49%)
2	4.9	79 (54%)	2	4.59	31 (21.2%
3	2.52	24 (23.1%			
Dunn's multiple comparison not performed as overall test data has not shown significant difference across samples			**Statistical Tes**t: Kruskal Wallace for comparison of means when >2 groups		





Lobular Inflammation	Mean CLDQ score	N (%)	Fibrosis	Mean Value	N (%)
0	4.79	27 (18.4%)	0	4.58	29 (19.7%)
1	5.00	69 (46.9%)	1	5.11	29 (19.7%)
2	4.38	49 (33.3%)	2	4.36	28 (19%)
3	3.03	2 (1.4%)	3	4.57	40 (27.2%)
Multiple comparison test Stage 2 versus stage 1 (4.38 versus 3.03, p=0.017)			4	4.53	21 (14.3%)

*__Statistical test__; dependence between histological parameters and CLDQ was measured by the Kruskal-Wallis test where $p<0.05$ was considered statistically significant

2.3.3.4. NASH and CLDQ scores

NASH (NAS score ≥4) was present in 62 patients. The clinico-demographic details of NASH versus Non-NASH group are summarised in **Table 2.9.** the NASH and non-NASH groups were similar across all measured parameters excluding BMI and AST, which were higher in the NASH group. As expected, more severe histological features were noted in the NASH group versus the non-NASH group. Higher CLDQ scores and lower ESS and FIS scores were recorded in the NAFL groups.

Table 2.9. Clinico-demographic characteristics NASH (NAS>4 versus Non-NASH)

Patient demographics and metabolic profile			
	NASH N=62	**Non-NASH N=85**	**P-Value**
Gender (Male)*	47(55%)	38 (45%)	0.529
Age	53 +/- 12	52 +/- 13	0.928
BMI	36 +/- 5	35 +/- 6	**0.039**
Obesity	59 (95%)	75 (88%)	0.144
T2DM*	38 (61%)	52 (61%)	0.989
ALT	104+/- 72	84 +/- 53	0.142
AST	72 +/-41	52 +/- 22	**0.003**
Albumin	44 +/-3	45 +/- 3	0.260
Platelet count	239 +/-71	256 +/- 85	0.299
Cholesterol	5.4 +/-1.3	5.2 +/- 1.3	0.421
Histology			
Steatosis (0/1/2/3)	0/1/35/36	1/32/44/8	**<0.0001**
Ballooning (0/1/2)	0/32/30	44/40/1	**<0.0001**
Lobular inflammation (0/1/2/3)	0/15/45/2	27/54/4/	**<0.0001**
Fibrosis (0/1/2/3/4)	0/6/16/30/10	29/23/12/10/11	**<0.0001**
Patient Reported Outcomes			
CLDQ	4.49 +/- 1.2	4.90+/- 1.35	**0.038**
FIS	86.93 +/- 33.23	74.08 +/- 33.54	**0.010**
ESS	8.59 +/- 5.3	6.83 +/- 4.27	**0.010**
BIV2	14.39+/-10.4	13.28 +/- 11.37	0.084

BMI= body mass index; obesity defined as BMI>30kg/m^2; ALT = Alanine aminotransferase; AST= aspartate aminotransferase

Data are expressed as *number (percentage) or #mean (standard deviation). *Statistical test; Mann-Whitney U rank test to compare continuous variable; chi square to compare categorical variables *Binary categorical variables used paired sample t-test.

NASH was associated with was a significantly lower HRQL compared to Non-NASH counterparts (mean (SD): 4.49 +/- 1.2 vs, 4.9 +/- 1.3; p=0.038). Additionally, patients with NASH scored lower in all CLDQ subscales but did not reach statistical significance for "abdominal symptoms", "emotional function" and "worry". **(Table 2.10)**

Table 2.10. Comparison of Health-related quality of life in NAFLD and NASH

	Total (n=147)	NAFL (n=85)	NASH (n= 62)	p-value
CLDQ total score	4.73 +/- 1.3	4.90 +/- 1.3	4.49+/- 1.2	***0.038***
Abdominal symptoms	5.24 +/- 1.6	5.34 +/-1.7	5.10 +/-1.5	0.259
Fatigue	4.12 +/- 1.6	4.41 +/-1.5	3.73 +/-1.6	***0.010***
Systemic symptoms	3.94 +/-1.1	4.12 +/-1.1	3.70 +/-1.1	***0.018***
Activity	5.2 +/- 1.5	5.47 +/-1.6	4.87 +/-1.3	***0.004***
Emotional functioning	4.6 +/- 1.6	4.66 +/-1.7	4.42 +/-1.6	0.342
Worry	4.91 +/- 1.6	5.08 +/-1.7	4.68 +/-1.6	0.062
*Data are expressed as means and standard deviations; statistical test; Mann-Whitney U test				

2.3.3.5. Differences in health-related QoL in Europe

This study formed part of a collaborative study with our colleagues in the University Centre of the Johannes Gutenberg-University Mainz, Germany and the University Hospital of Seville as part of the prospectively enrolling European NAFLD Registry. Additional CLDQ data, with each respective institute's permission, has been included in this thesis enabling a brief comparison between the three enrolling European countries to be performed. A full manuscript describing these differences has been published by Huber et al (274)The clinico-demographic details of liver function and histological features for each country are demonstrated in **Table 2.11.** A number of significant differences existed between the groups in the categories of age, BMI, T2DM, Hypertension, ALT, AST, Albumin and HBA1c.

Table 2.11. Demographic data, characteristics of liver function, and histological features in the UK, Germany and Spain

	Total (n=297)	UK cohort (n=147)	German cohort (n=133)	Spanish cohort (n=17)	P-Value
Male	162 (53.3)	85 (56.5)	69 (51.9)	6 (35.3)	0.82
Age (range)	54 (17-77)	53 (17-77)	53 (21-75)	61 (33-74)	**0.05**
BMI	33.3 (30.0; 37.5)	35 (31.6; 38.7)	31.9 (28.7; 36.3)	31.2 (27.3; 37.0)	**<0.0001**
Obesity	228 (75.0)	134 (91.0)	85 (63.9)	10 (58.8)	**<0.0001**
T2DM	156 (51.3)	90 (61.0)	52 (39.1)	9 (52.9)	**<0.001**
Hypertension	203 (66.8)	87 (56.5)	102 (76.7)	14 (82.4)	**<0.001**
Hyperlipidemia	177 (58.2)	88 (57.1)	83 (62.4)	6 (35.3)	0.07
ALT	73 (48; 110)	93 (48; 109)	81 (51; 110)	33 (24; 61)	**<0.001**
AST	50 (36; 69)	60 (38; 71)	51 (37; 68)	29 (24; 54)	**<0.001**
γ-GT	84 (56; 162)	92 (59; 164)	80 (53; 161)	82 (45; 223)	0.5
Albumin	43 (40; 45)	44 (43; 47)	41 (39; 43)	45 (43; 47)	**<0.001**
Platelet count	233 (183; 283)	249 (190; 296)	226 (183; 270)	190 (176; 228)	0.05
HbA1c	6.1 (5.5; 7.1)	6.3 (5.75; 7.6)	5.7 (5.3; 6.3)	6.5 (6.2; 7.4)	**<0.001**
			Histological findings		
NASH (NAS>4)	163 (66%)	62 (42%)	89 (66.9)	12 (70.6)	0.77
Steatosis 0/1/2/3	1/99/152/44	1/33/79/34	0/58/67/7	0/8/6/3	**<0.001**
Ballooning 0/1/2	82/163/51	44/72/31	34/81/17	4/10/3	0.26
Lobular inflammation 0/1/2/3	63/163/67/3	27/69/49/2	32/87/12/1	4/7/6/0	**<0.001**
Fibrosis 0/1/2/3/4	36/74/67/82/45	29/29/28/40/21	5/43/36/37/12	2/2/3/5/5	**<0.001**

BMI= body mass index; obesity defined as BMI>30kg/m^2; ALT = Alanine aminotransferase; AST= aspartate aminotransferase

Data are expressed as *number (percentage) or #median (25,75th percentiles)

*Statistical test Kruskall Wallace test to compare continuous variable between groups (>2); chi square to compare categorical variables

There were also significant differences in CLDQ scores between the 3 European countries **Table 2.12.**

Table 2.12. Comparison of Health-related quality of life in sub-cohorts

Parameter	Total (n=304)	UK cohort (n=154)	German cohort (n=133)	Spanish cohort (n=17)	P-Value
CLDQ overall score	4.99 (±1.2)	4.73 +/- 1.3	5.27 (±1.1)	5.14 (±1.1)	**<0.01**
Abdominal symptoms	5.33 (±1.6)	5.24 +/- 1.6	5.51 (±1.5)	4.76 (±1.6)	0.12
Fatigue	4.31 (±1.6)	4.12 +/- 1.6	4.48 (±1.5)	4..64 (±1.7)	0.09
Systemic symptoms	5.09 (±1.3)	3.94 +/-1.1	5.37 (±1.2)	5.35 (±1.2)	**<0.01**
Activity	5.43 (±1.4)	5.2 +/- 1.5	5.73 (±1.2)	5.12 (±1.4)	**<0.01**
Emotional functioning	4.93 (±1.5)	4.6 +/- 1.6	5.30 (±1.3)	5.32 (±1.4)	**<0.001**
Worry	5.18 (±1.5)	4.91 +/- 1.6	5.46 (±1.3)	5.38 (±1.1)	**<0.01**

*Data are expressed as means and standard deviations; statistical test; Kruskal Wallace

Compared to Germany and Spain, the UK exhibited the lowest QoL scores. In the UK, lowest scores were observed in the subcategory “systemic symptoms”, while in both Germany and Spain the lowest scores were recorded in the subcategory “fatigue”. No differences were observed in the subcategories “fatigue” and “abdominal symptoms” between countries.

Overall, the general QoL data extracted from the CLDQ assessment has shown us that the lowest scores have been recorded in the subcategories “fatigue”, “systemic symptoms” and “emotional functioning”.

Thematic categories of fatigue and depression were explored further using validated scales.

2.3.4. The relationship between fatigue, depression, QoL and NAFLD

There are strong negative linear correlations between CLDQ scores, fatigue (FIS and ESS) and depression (BIV2) scores underlining the fact that these factors lead to a significantly impaired QoL. A strong positive linear correlation exists between patient reported fatigue (FIS), hypersomnolence and depression. Sub-dividing the chronic liver disease cohort into subjects with NASH (n=62) and NAFL (n=85), the findings continued to significantly trend in the same direction. ***(Table 2.13)***

Table 2.13 Correlations CLDQ with patient reported fatigue and hypersomnolence in patients with NASH and NAFL.

		Total Cohort (n=147)		**NAFL (n=85)**		**NASH (n=62)**	
		Rs	P-Value	Rs	P-Value	Rs	P-Value
CLDQ	**FIS**	-0.801	<0.0001	-0.797	<0.0001	-0.790	<0.0001
	ESS	-0.470	<0.0001	-0.467	<0.0001	-0.432	0.001
	BIV2	-0.744	<0.0001	-0.749	<0.0001	-0.731	<0.0001
FIS	**ESS**	0.541	<0.0001	0.616	<0.0001	0.408	0.001
	BIV2	0.760	<0.0001	0.790	<0.0001	0.722	<0.0001
ESS	**BIV2**	0.430	<0.0001	0.498	<0.0001	0.353	0.006

*CLDQ= Chronic Liver Disease Questionnaire; FIS= Fatigue Impact Score; ESS= Epworth Sleepiness Score; BIV2= Beck Inventory version 2.
*Statisitical test; Spearman's correlation coefficient (Rs)

2.3.4.1. Fatigue and NAFLD disease severity

A total of 62 patients had NASH. There was a significant trend toward higher FIS scores in patients with NASH compared to those with NAFL (87 +/-33 vs 74 +/- 34, p=0.010). This trend was maintained in the sub-categories "physical" and "social". 86 patients had mild fibrosis. There was a trend toward higher FIS scores in all categories in the severe fibrosis group however this did not reach statistical significance. 21 patients had cirrhosis. Again, there was a trend toward higher FIS scores in the cirrhotic group however this did not reach statistical significance. **(Table 2.14)**

Table 2.14 FIS subcategories and NAFLD Disease Severity

FIS	Total Cohort (n=147)	NASH (n=62)	NAFL (n=85)	P-Value	Mild Fibrosis F012 (n=86)	Severe Fibrosis F34 (n=61)	P-Value	FO123	Cirrhosis F4 (n=21)	P-Value
Total Score	79 +/- 34	87 +/-33	74 +/- 34	**0.010**	76 +/- 34	85 +/- 34	0.108	79 +/- 34	81 +/- 32	0.798
Cognitive	19 +/- 9	20 +/- 10	18 +/- 8	0.240	19 +/- 9	20 +/- 10	0.656	19 +/- 9	18 +/- 9	0.813
Physical	23 +/- 10	26 +/- 10	20 +/- 10	**0.001**	21 +/- 10	25 +/- 10	**0.020**	23 +/- 10	22 +/- 11	0.910
Social	37 +/- 17	40 +/- 17	36 +/- 18	**0.045**	36 +/- 17	39 +/- 18	0.259	37 +/- 17	36 +/- 18	0.921

*Data are expressed as means and standard deviations; statistical test; Mann-Whitney U test

To look for potential mechanisms of fatigue in NAFLD, correlations between subcategories of the FIS score and NAFLD patient liver histology status were assessed **(Table 2.15).** Significant correlations existed between Total FIS score and lobular inflammation (Rs = 0.228, p=0.006). Grade of lobular inflammation significantly correlated with all sub-domains of the FIS. Ballooning on liver biopsy correlated with the "physical" subcategory. There was no correlation between pathological hypersomnolence or the presence of clinical depression with NAFLD disease severity**.**

Table 2.15 Correlation of patient reported fatigue and severe depression with NAFLD Histology

	Total FIS Score	P-value	Cognitive	P-value	Physical	P-value	Social	P-value	ESS >10	P-Value	BIV2 >20	P-Value
Steatosis	0.42	0.617	-0.05	0.550	0.106	0.200	0.030	0.719	0.029	0.745	-0.022	0.789
Ballooning	0.133	0.109	0.061	0.460	**0.190**	**0.021**	0.073	0.377	0.70	0.425	-0.021	0.804
Lobular Inflammation	**0.228**	**0.006**	0.125	0.133	**0.290**	**<0.0001**	**0.205**	**0.013**	0.150	0.087	0.117	0.158
Fibrosis	0.090	0.282	0.013	0.876	0.130	0.115	0.050	0.551	0.073	0.406	-0.010	0.901

*Statistical test; Spearmans correlation co-efficient (Rs)

No significant correlations existed between FIS scores and NAFLD patient biochemistry, gender, age or the presence of obesity. A moderate positive correlation existed between FIS scores and BMI **(Table 2.16).**

Table 2.16 Correlation of patient demographics and biological data with patient reported fatigue

	UK cohort (n=147)	**Correlation**	**P-Value**
Male*	85 (56.5)	0.114	0.172
Age (range)	53 (17-77)	-0.087	0.295
BMI	35 (31.6; 38.7)	0.225	**0.006**
Obesity	134 (91.0)	0.114	0.170
T2DM *	90 (61.0)	0.067	0.419
ALT	93 (48; 109)	0.010	0.908
AST	60 (38; 71)	0.119	0.163
Albumin	44 (43; 47)	-0.066	0.429
Platelet count	233 (183; 283)	0.109	0.193

*Statistical test; Spearmans correlation co-efficient (Rs)
**Paired t-test for binary categorical variables

2.3.5. Exploring the potential confounding effect of depression on CLDQ and FIS scores

In order to consider the potential confounding of depression, patients with moderate or severe depression were excluded. This generated a cohort of subjects (n=113) with no or mild depression. For fatigue, BMI and Lobular inflammation correlated with FIS scores in a positive, linear and significant manner. For CLDQ, male gender and BMI correlation with CLDQ scores in a negative, linear and significant manner. This trends were the same as those observed in the complete cohort including patients with severe depression. **(Table 2.17).**

Table 2.17 Correlation of patient reported fatigue and CLDQ with demographic, biological and histological data in a cohort of patients with mild/no depression

	UK cohort (n=113)	**Correlation with FIS**	**P-Value**	**Correlation with CLDQ**	**P-Value**
Male	68 (60%)	0.079	0.405	-0.323	**<0.0001**
Age (range)	55 +/- 12	-0.001	0.992	0.007	0.945
BMI	35 +/- 5	0.222	**0.019**	-0.320	**0.001**
Obesity	102 (90%)	0.110	0.250	-0.186	0.048
T2DM	67 (59%)	0.033	0.732	-0.078	0.412
ALT	93 +/- 64	0.040	0.676	-0.042	0.662
AST	61 +/- 35	0.139	0.156	-0.182	0.061
Albumin	44 +/- 3	-0.146	0.101	0.181	0.055
Platelet count	241 +/- 75	-0.004	0.971	-0.034	0.722
Steatosis	1/26/58/28	0.074	0.438	-0.107	0.261
Ballooning	34/54/25	0.158	0.095	-0.128	0.179
Lobular Inflammation	20/59/33/1	0.231	**0.014**	-0.180	0.056
Fibrosis	22/23/21/30/17	0.126	0.184	-0.122	0.198

*Statistical test; Spearmans correlation co-efficient (Rs)
**Paired t-test for categorical variables

2.3.6. The influence of liver histology on the presence of pathological depression, fatigue and QoL impairment

To determine whether elevated ESS, FIS, BIV2 or CLDQ scores were influenced by liver disease severity, a one-way ANCOVA was conducted to determine if having significant hypersomnolence, Fatigue, depression, or impaired HRQL was related to liver disease histology severity. Likely confounding factors were included as covariates in each analysis **(Table 2.18).**

Epworth Sleepiness scale. NAFLD histology did not influence the presence or absence of significant hypersomnolence.

Fatigue Impact Score NAFLD histology did not influence patient reported fatigue.

Beck Depression Inventory. NAFLD disease histological parameters did not influence the presence of moderate to severe depression.

Chronic Liver Disease Questionnaire; QoL scores were influenced by liver fibrosis stage ($F=41.075$, $p<0.0001$). The effect size was large 0.814. The most significant message from this stringent analysis was that CLDQ scores are influenced by fibrosis stage when controlling for fatigue, mental health and the other histological parameters not being assessed. Mental health was surprisingly not influenced by NAFLD histology when controlling for fatigue and QoL.

Table 2.18 ANCOVA Analysis: Fatigue, Hypersomnolence, Depression and HRQL and relationship to NAFLD histology severity.			
	F	**P-Value**	**Eta Squared- Effect Size**
Epworth Sleepiness Scale ***controlling for co-variates FIS, HRQL, BIV2 and BMI and histological parameters not being assessed**			
Steatosis			
Total ESS	0.834	0.663	0.130
Ballooning			
Total ESS	0.828	0.669	0.129
Lobular inflammation			
Total ESS	1.416	0.135	0.202
Fibrosis			
Total ESS	0.889	0.597	0.137
Fatigue Impact Score ***controlling for co-variates ESS, HRQL, BIV2 and BMI and histological parameters not being assessed**			
Steatosis			
Total FIS	0.676	0.939	0.507
Ballooning			
Total FIS	0.917	0.638	0.582
Lobular inflammation			
Total FIS	1.528	0.056	0.699
Fibrosis			
Total FIS	0.985	0.531	0.6
Beck's Depression Inventory ***controlling for co-variates FIS, ESS, HRQL, BMI and histological parameters not being assessed**			
Steatosis			
Total BIV2	0.541	0.980	0.180
Ballooning			
Total BIV2	0.947	0.561	0.277
Lobular inflammation			
Total BIV2	1.411	0.098	0.363
Fibrosis			
Total BIV2	0.988	0.502	0.285
Chronic Liver Disease Questionnaire ***controlling for co-variates FIS, ESS, BMI**			
Steatosis			
Total CLDQ	1.834	**0.019**	0.808
Ballooning			
Total CLDQ	1.160	0.310	0.726
Lobular inflammation			
Total CLDQ	0.849	0.738	0.660
Fibrosis			
Total CLDQ	1.910	**0.014**	0.814
ESS= Epworth sleepiness scale; FIS= fatigue Impact Scale; BIV2= Beck Depression Inventory version 2; CLDQ= Chronic Liver Disease Questionnaire *Statistical test; ANCOVA F =Test statistic, $p<0.05$ statistically significant			

2.3.7. Depression and NAFLD- Further Exploration

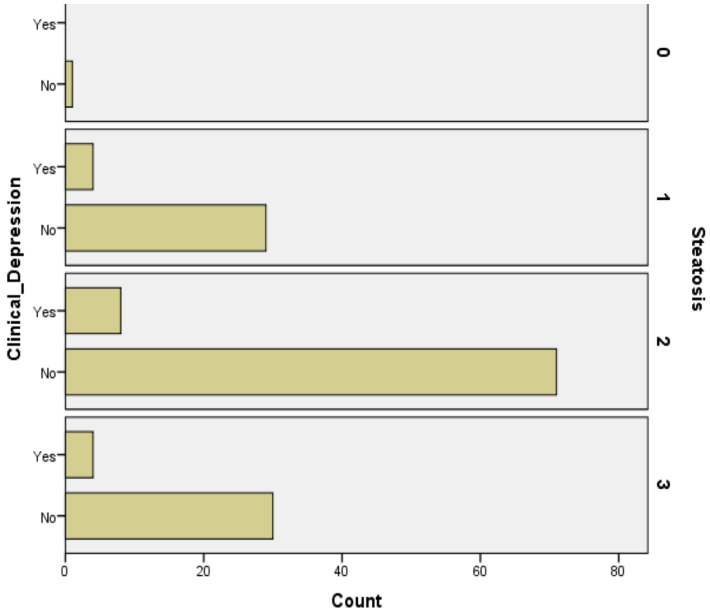
In this study, no clear relationships have been suggested regarding depression and NAFLD. A univariate analysis was conducted to see if patient demographics or biological data influenced the presence of severe depression in NAFLD. **(Table 2.19).** For age in years, risk of moderate to severe depression decreases as the number of years escalates. For platelet count, for each unit increase in platelets the risk of severe/moderate depression increases by an OR of 1.005. count. Liver histology odds ratios did not reach statistical significance.

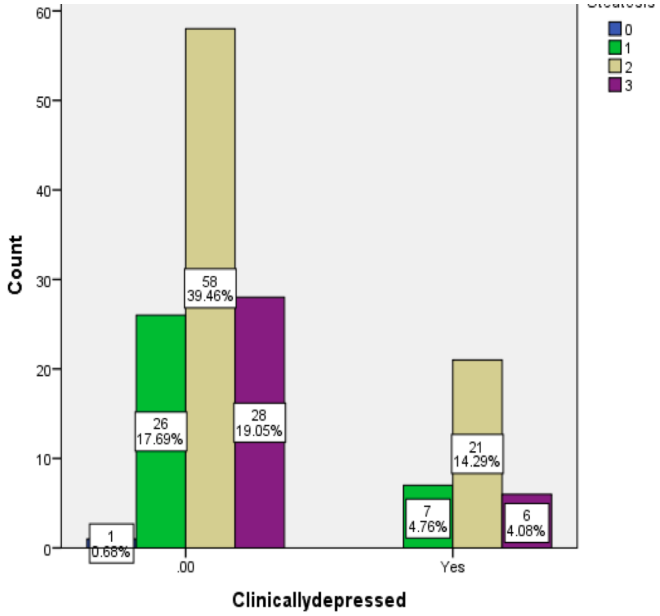
Table 2.19 Variables Associated with the Presence of moderate/severe depression in NAFLD

	Univariate		
Variable	**Odds Ratio**	**95% CI**	**p-value**
Age	0.960	0.931-0.989	**0.007**
Gender	1.511	0.699-3.266	0.294
BMI	1.065	0.991-1.145	0.0891
T2DM	1.436	0.638-3.229	0.382
ALT	1.000	0.994-1.006	0.988
AST	0.999	0.987-1.011	0.895
Albumin	1.068	0.948-1.203	0.281
Platelets	1.005	1.000-1.010	**0.039**
Steatosis	0.941	0.542-1.633	0.941
Ballooning	0.927	0.539-1.594	0.784
Lobular Inflammation	1.433	0.840-2.444	0.187
Fibrosis	0.983	0.740-1.304	0.903
Severe Fibrosis	0.983	0.451-2.142	0.966
Cirrhosis	0.753	0.235-2.411	0.633
NASH	1.295	0.599-2.799	0.511
Obesity	1.725	0.363-8.196	0.493
NASH= NAS score >4: Obesity = BMI> 30kg/m^2 Statistical test; Univariate analysis			

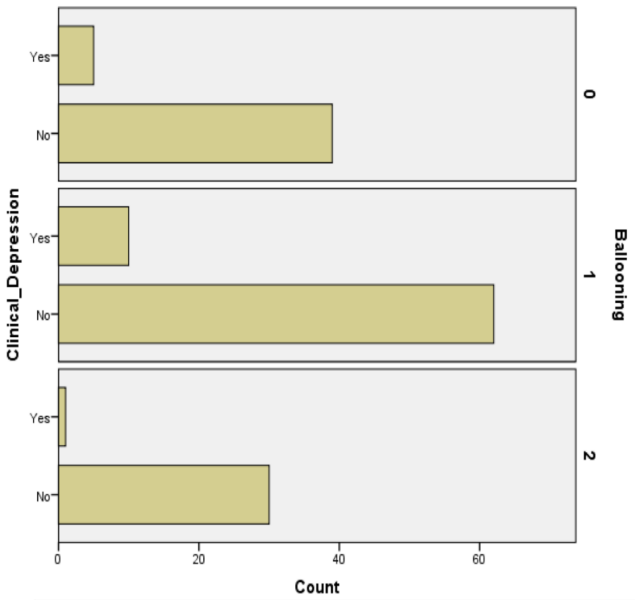
A subtle trend towards worsening NAFLD histology was observed in patients with moderate to severe depression (Becks Inventory Depression Version scores >20) but did not reach statistical significance (P>0.05) **(Figure 2.3)**

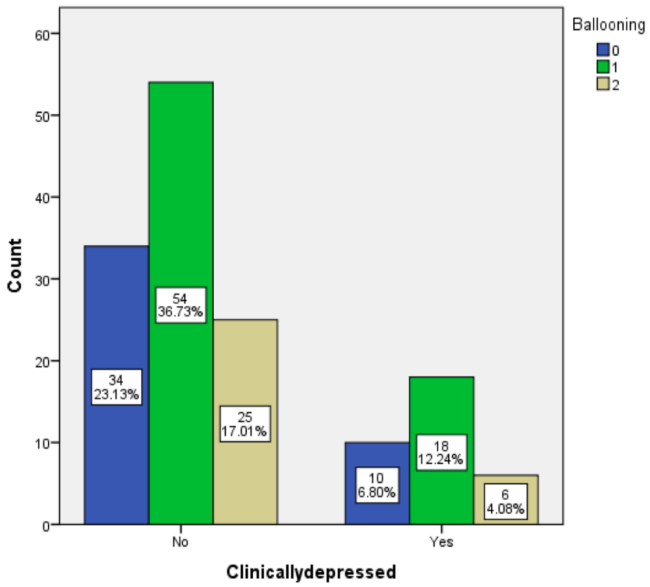
Figure 2.3 Trends, liver histology and depression



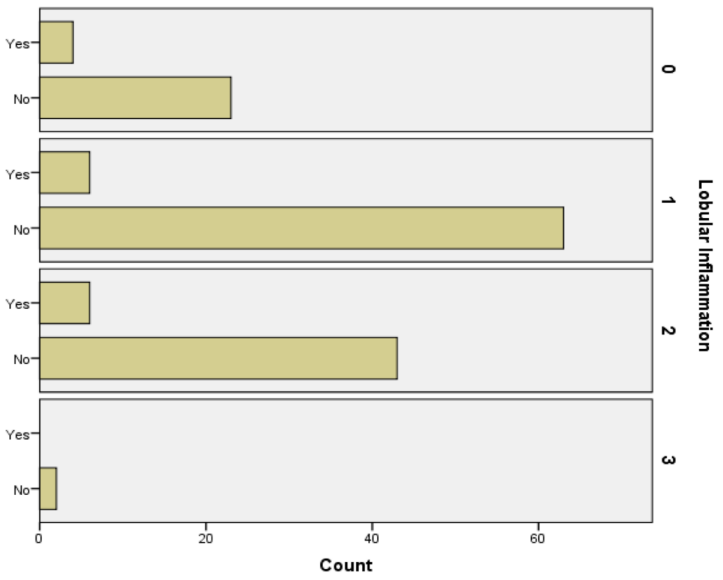


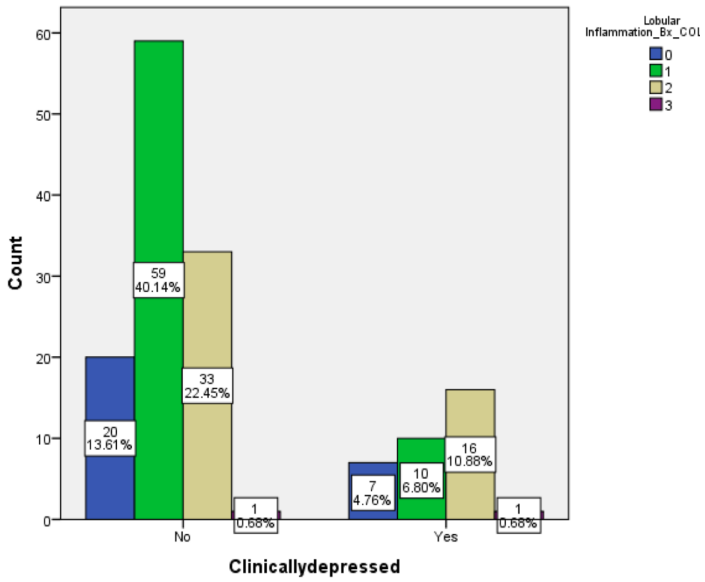
Pearson's Chi-Squared test X^2	P-value
1.474	0.688
No relationship observed between moderate – severe depression and steatosis	



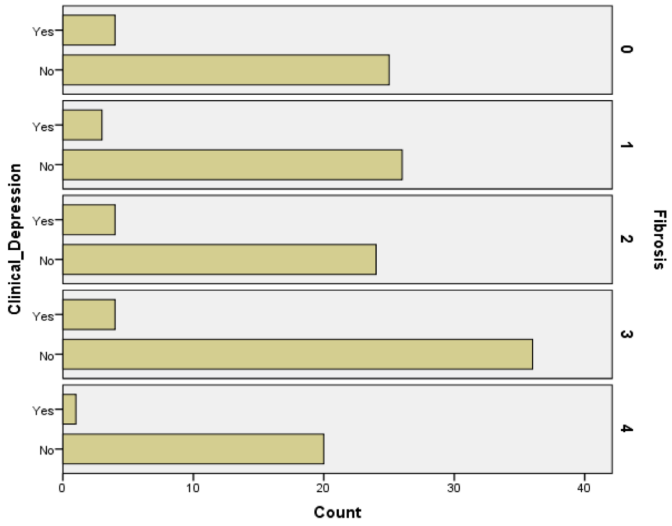


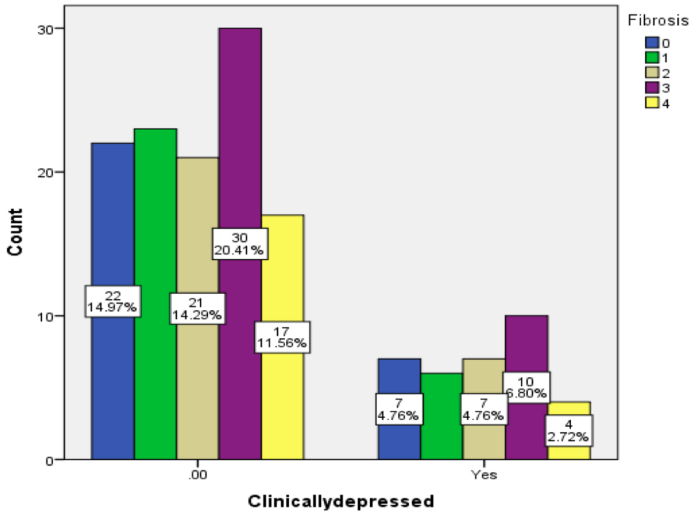
Pearson's Chi-Squared test X^2	P-value
3.94	0.821
No relationship observed between moderate – severe depression and Ballooning	





Pearson's Chi-Squared test X^2	P-value
6.325	0.097
No relationship observed between moderate – severe depression and Lobular Inflammation	





Pearson's Chi-Squared test X^2	P-value
0.444	0.097
No relationship observed between moderate – severe depression and fibrosis	

2.4. DISCUSSION

The literature supports a multifactorial model of HRQL impairment in advanced liver disease (246). In this study, a combination of disease specific measures (CLDQ), with additional data provided by validated domain specific questionnaires was performed. Generic scales were not utilised in this study, however data to date has shown good correlation between generic and disease specific scales enabling us to compare our findings to both CLDQ and SF-36 scales currently reported in the literature (101, 242, 275). Our subject selection demonstrated sample heterogeneity in terms of histology and importantly our group was homogeneous with regard to medical co-morbidities and patient demographics which can significantly influence QoL outcomes. Overall, this study showed that a substantial QoL symptom burden exists in patients with NAFLD.

This study was conducted cognisant of the fact that PROs are gaining momentum as clinical trial end-points. In clinical trials registered on ClinicalTrials.gov, the use of PRO endpoints between 2004 and 2007 increased from 14-27% (276), with an Australian analysis reporting that 45% of trials registered between 2005 to 2017 included PROs as endpoints (277). In recognition of this trend, in 2009 the FDA compiled guidelines to streamline the FDA's review of PRO measures and associated clinical trial data (105).

2.4.1. Impaired QoL in NAFLD

General observations the mean CLDQ score recorded in this study was 4.72, consistent with a self-reported impaired QoL. This reflects the current trends demonstrated in US studies with 2 research groups reporting that in QoL assessments, NAFLD patients score lowest in the domains of general health, vitality, physical health and body pain (102, 278). When CLDQ scores from this study were compared to data obtained from other published sources (269), these levels were significantly lower than normal healthy control populations and in chronic liver disease patients with HBV ($p=<0.0001$). NAFLD QoL scores were also significantly lower than in the PBC or HCV populations. This slightly contradicted a US study (n=150) also employing the CLDQ scale in CLD. Alt et al reported a mean CLDQ of 5.35 for NAFLD patients compared to CLD patients with non-viral disease and concluded that no aetiology driven reason for differences in CLDQ measurements existed (261). The CLDQ findings in this study also challenge another currently accepted dogma. It is generally

acknowledged that HRQL can be influenced by national and social factors however, despite this recent studies have consistently shown that inter-variability between countries is in most cases negligible (279). However, in this study compared to their European counterparts in Spain and Germany, UK NAFLD subjects had the lowest CLDQ scores (4.73 versus 5.14 versus 5.27 respectively, $p<0.0001$) with the UK reporting the lowest scores in all subdomains except for in the abdominal and fatigue categories.

CLDQ and clinico-demographic patient details Preliminary exploration of CLDQ scores revealed findings consistent with those reported in the literature. A study by David et al from 2009 has suggested that older age, female gender, lower educational level and lower socio-economic levels strongly correlate with QoL scores and their sub-domains (280). Attempts to clarify these associations would provide a better foundation to detect vulnerable populations and provide opportunistic care (243). In this study, CLDQ scores demonstrated a moderate, negative linear correlation with male gender, BMI, lobular inflammation on liver biopsy and the presence of NASH. Subgroup analysis demonstrated that women scored significantly lower on all subcategories in the CLDQ. This effect remained significant despite homogeneity in disease variants between male and female groups. This finding is not specific to NAFLD and is also observed in HCV-HIV co-infected patients (281). Advancing age or the presence of T2DM did not significantly influence total CLDQ scores however regarding T2DM, patients with the diagnosis scored significantly lower in the subdomains fatigue, worry and activity compared to non-diabetics. Regarding BMI, obese subjects had significantly lower CLDQ scores compared to non-obese counterparts (4.66 versus 5.64). NASH, characterised by lobular inflammation and ballooning has been linked to elevated cytokine levels and markers of systemic inflammation associated with metabolic syndrome components. It is interesting to note that such inflammatory markers are reported to promote depressive symptoms and perhaps lead to poorer CLDQ scores (282-284).

CLDQ and NAFLD histology Controlling for significant biological co-variants, this study demonstrated that lobular inflammation grade influenced CLDQ scores ($F=3.802$, $p=0.012$). Total CLDQ scores correlated linearly, significantly and negatively with the grade of lobular

inflammation overall and in 5/6 subdomains. On subgroup analysis, CLDQ scores were lower in subjects with NASH (4.49 versus 4.9, p=0.038) and this extended to the subdomains fatigue, systemic symptoms and activity. Hepatocyte apoptosis is synonymous with NASH, and in a study investigating an apoptosis biomarker (Cytokeratin 18), a small negative correlation was observed between CK18 levels and CLDQ scores (261). These findings support the possible role of liver-protective therapies for QoL improvement in chronic liver disease. Accordingly, SF-36 data was available from the PIVENS trial, however in this 96 week trial QoL scores did not significantly differ between interventional arms (138). Regarding fibrosis, reports from a study performed over a decade ago report lower HRQL in a US population with cirrhosis using the short form-36 HRQL (128, 259). Hepatic fibrosis stage reflects the summation of liver injury that occurs over the lifetime of the NAFLD. Regarding fibrosis in NASH, this study has demonstrated that although a trend exists for lower scores in cirrhotics it is not statistically significant. Furthermore, comparing groups with mild and severe fibrosis, significant differences between mild and severe fibrotic subjects only existed in the subdomains fatigue, activity and worry. However, following an ANCOVA analysis controlling for Fatigue scores (FIS and ESS), depression (BIV2) and BMI, change in fibrosis stage was found to influence total CLDQ scores with a large effect size.

Lowest CLDQ subdomain scores NAFLD subjects in this study scores lowest in the subdomains "systemic symptoms" (3.94), fatigue (4.12) and emotional functioning (4.6). Compared to PBC, HBV, HCV and normative data, the NAFLD population maintained the lowest scores in subdomain "systemic symptoms'. Pro-inflammatory cytokines such as TNF-alpha and interleukin-6 are believed to induce such "systemic" symptoms commonly observed in depressive disorders (285).

Rationale for thematic categories driven research derived from CLDQ data Given that fatigue (4.12) and emotional functioning (4.6) reported the lowest scores and validated scales exist for both, these were the subdomains considered for further analysis. The decision was supported by the findings of significant negative linear correlations between total CLDQ scores and fatigue and depression scales. To complement quantitative QoL data interpretation, it is useful to consider qualitative study findings (286). A NAFLD focus group

reported symptoms and impacts related to NASH. The core disease related concepts achieving saturation included fatigue, pain/discomfort, abdominal issues, sleep, social/emotional issues, sweating, and concentration. This mirrors the themes and findings reported in the CLDQ data obtained in this study. Again, the most prominent reported symptom was fatigue experienced by 67% of patients and reported across 89% of sessions. (286).

2.4.2. Fatigue and NAFLD

The available literature identifies that fatigue is commonly reported by NAFLD patients and is strongly correlated with a worse QoL (102, 268, 287). Fatigue, although a non-specific, subjective symptom warrants clinician's attention due to the negative impact it has on patient's well-being (243).

General observations the mean FIS score in the study cohort was 79. 100% of subjects had significant fatigue with scores >29. This was mirrored by a mean score of 8 in the Epworth sleepiness score with 22% of subjects having significant hypersomnolence. Positive linear correlations exist between FIS scores, hypersomnolence and depression indices indicating that perceived fatigue contributes to both.

Fatigue subdomain in CLDQ Fatigue was the lowest scoring subdomain in the CLDQ. NAFLD patients had a mean score of 4.12, significantly lower than the control group (5.0) and comparable to the PBC, HCV and HBV groups at 3.7, 4.0 and 5.6 respectively. In Europe, patients scored lowest in the fatigue subcategory (4.31), with the lowest reported level in the UK (4.12). The best fatigue scores were reported in Spain (5.12). The role of the sleep-wake cycle in the pathogenesis of NAFLD has been studied in murine models and is influenced by number of daylight hours. This may be significant in the discrepancy observed between fatigue scores in the UK and Spain where impaired sleep duration and sleep quality have been identified as risk factors for NAFLD in the middle-aged population (288). In the fatigue subdomain of CLDQ, Ballooning and lobular inflammation significantly negatively correlated with fatigue score (R_s= -0.183, p= 0.026 and R_s= -0.275, p= 0.001, respectively) indicating that with increasing NAFLD severity, fatigue increases and QoL worsens.

FIS and clinico-demographic patient details FIS correlated with BMI only (Rs=0.225, p=0.006) supporting the stereotype of the "fat, tired person". Studies have shown that sleep duration is associated with weight and BMI in patients with NAFLD (288) NAFLD studies have shown that compared to healthy controls, time to fall asleep was significantly prolonged, sleep duration was shortened and quality of sleep was poor accounting for an increase in perceived tiredness (288). Regarding patient biochemistry, in this study, no correlation was observed between fatigue (ESS and FIS scales) and liver enzymes. These findings were in contrast to a study showing that daytime sleepiness correlated with transaminases (288). Current evidence would suggest that only one study has linked daytime sleepiness with biochemical parameters in NAFLD (288) although it has also been demonstrated in rat models of REM sleep deprivation (289).

FIS and NAFLD histology Grade of lobular inflammation significantly influenced FIS scores (F=4.908, p=0.012) while grade of fibrosis significantly influencing hypersomnolence (F=2.470, p=0.049). These findings have implications for the clinical management of NAFLD as it is possible to infer that by employing treatments that target steatohepatitis and fibrosis, it is possible to improve the symptom of fatigue. FIS scores as predicted, are significantly higher in the NASH versus NAFL group (87 versus 74, p=0.010). FIS scores were not different between mild and severe fibrosis or in cirrhosis. In NAFLD, it is suspected that hepatic clearance mechanisms may be sub-optimal. Owing to impaired phagocytic function in hepatocytes, NAFLD patients have elevated lipopolysaccharide levels in the systemic circulation of NAFLD patients which can induce cytokines potentially linked with fatigue, independent of liver fibrosis (290, 291).

Subdomains of the FIS Score Subdividing the cohort into NASH (NAS$\geq$4) versus NAFL, differences in the subdomains "physical", (p=0.001) and "social" (p=0.045) were significant. In the mild versus severe fibrosis groups, differences existed in the "physical" subdomain (0.020). No difference in FIS scores were observed for cirrhotics versus non-cirrhotics. Total FIS correlated positively and significantly with lobular inflammation (LI) (Rs = 0.1228, p=0.006). LI correlated with the "physical" and "social" subcategories (Rs= 0.290, p=<0.0001, Rs= 0.205, p=0.013), the "physical" subcategory also correlated with Ballooning (Rs= 0.190,

p= 0.021). A possible explanation for this finding is the evidence base that proposes that fatigue in NAFLD may be also be as a result of having a chronic disorder whereby inflammatory cytokines, reactive oxygen species and free fatty acids (all proven to be systemically elevated in NAFLD) act on extra-hepatic sites (muscle and brain) causing symptoms of fatigue independent of liver inflammation or fibrosis (248). Further supporting this hypothesis is a previous study demonstrating that fatigue can occur independently of cirrhosis but is influenced by markers of disease severity (biochemical or histological) and insulin resistance (292)

Role of OSA and hypothyroidism in fatigue in NAFLD A high incidence of hypothyroidism has been observed in NAFLD population versus control population versus matched population with other chronic liver diseases (15% versus 7.2% versus 7.3%)(293) and is a potential cause for fatigue in NAFLD. However, in this study, no significant correlation between TSH levels and fatigue (assessed using the Fatigue Impact Scale, p=0.4; effect size=0.02) or hypersomnolence (assessed with the Epworth Sleepiness Scale, p = 1.0; effect size<0.0000) was observed. ESS is considered to be the gold standard for the subjective measure of daytime sleepiness. A second biological explanation for increased fatigue in NAFLD is the presence of obstructive sleep apnoea which has an increased reported incidence in NAFLD (OSA) (294, 295). Sleep apnoea is a primary sleep disorder that has been described as a risk factor for NAFLD and has been associated with NASH and fibrosis (296). The diagnosis is usually confirmed by a specialist respiratory work-up upon receipt of an ESS score greater than 10 (297). However, in this study, the mean FIS is pathological at 80 while only 22% of subjects have a pathological ESS scores greater than 10. These findings would support the conclusion of a more general association of sleep disruption and NAFLD (like in PBC) rather than a subgroup effect alone (248).

2.4.3. Depression scores and NAFLD

General observations in this study cohort, the mean BIV2 score was 14. 23% of the study group had moderate to severe depression with scores >20. In Europe, the UK group scored the lowest in the "emotional functioning" and "worry" subcategories (synonymous with mental health), compared to their European counterparts (p= 0.01). Depression scores were

found to significantly correlate with FIS, ESS and CLDQ (Rs= 0.760, 0.430, -0.744; p <0.0001) suggesting that depression plays a significant role in the NAFLD symptom burden.

BIV2 scores and patient clinico-demographic details Platelet count was significant on univariate analysis as a variable predictive of moderate to severe depression (OR= 1.010, p=0.003). However, the OR reported would suggest and elevated platelet count rather than a reduced platelet could would increase the likelihood of depression. Reduced platelet count is an indicator of advanced liver disease and liver disease progression has been linked with the mental component of QoL (100, 278, 280). Depressive symptoms may be explained by the degree of physical impairment associated with cirrhosis versus NASH. The increased mental health effects in cirrhotics are thought to be mediated by the occurrence of end-stage liver disease complications and treatment plan uncertainties (243).

BIV2 scores and histology BIV2 scores did not significantly correlate with steatosis, ballooning, lobular inflammation or fibrosis (p>0.05). Furthermore, uncontrolled, depressive symptoms were not significantly different between the NASH and NAFLD group (14 versus 13, p=0.084). ANCOVA analysis controlling for FIS, ESS, HRQL and BMI showed that depression was not influenced by NAFLD disease severity However, depressed mood has been shown to be associated with high levels of inflammatory markers, suggesting that depressed mood is causing and/or is caused by systemic inflammation (285). A research group in the US has hypothesised that in NAFLD, depressive symptoms promote weight accumulation, which in turn activates an inflammatory response through two distinct pathways: expanded adipose tissue release of interleukin-6 and leptin-induced upregulation of interleukin-6 release by white blood cells. This refutes a recognised sickness behaviour model in which the inflammatory molecules arising from expanded adipose tissue promote depressive symptoms (298). Although, not supported by findings in this study, depressive symptoms and NAFLD histology offers a potential avenue for treatment. In humans, serotonin degradation by monoamine oxidase A (MAO-A) is a source of reactive oxygen species (ROS) which mediate hepatocellular injury in NASH. MAO-A expression is found to be up-regulated significantly in human NASH, thus offering the possibility that serotonin plays a role in the pathogenesis of steatohepatitis, and therefore might represent a novel

target for the prevention and treatment of NASH (256). Larger prospective, basic science studies are need to fully explore this given the convincing results in murine models and the widespread availability of approved MAO-Inhibitor compounds available.

CLDQ scores- relevant subdomains the "emotional" subcategory of the CLDQ value (4.6) was lower than the normal control population (6.0, (p=<0.0001) and comparable to the PBC (4.7) and HCV (4.6) populations. This is keeping with the literature where other liver disease aetiologies are reported to have worse findings in relation to mental health, such as the HCV subgroup (299). However, it should be noted that in studies large enough to perform meaningful multivariate analysis; outcomes would suggest that the diagnosis of NAFLD independently predicts mental and physical QoL after controlling for demographic variables and co-morbid conditions that might affect a person's well-being (100, 103).

Worsening scores in the mental health subcategories of the CLDQ may also be explained by looking at patient metabolic profiles.

2.4.4. Metabolic co-morbidities and QoL

General observations in this cohort, 61% had T2DM, 57% had hypertension, 57% had hyperlipidaemia and 91% were classified as obese with a mean BMI of 35 +/- 5 kg/m2. To date, several studies have attempted to summarise the contributions of metabolic syndrome components to patient QoL and the results have been inconclusive (102, 278, 280).

CLDQ and patient metabolic profile Total CLDQ scores did not correlate with the presence of T2DM but correlated significantly with BMI (Rs=-0.307, p=<0.0001).

Obesity In obese patients, CLDQ scores were lower compared to non-obese subjects (4.66 versus 5.44, p=0.036). This trend extended to all subdomains except abdominal symptoms. Although the majority of NAFLD patients are overweight, a cohort of lean NAFLD patients exists, where this category of hepatic steatosis represents a distinct subgroup which may provide useful insights for exploring obesity and metabolism abnormalities in QoL (243, 300).

T2DM CLDQ scores were also lower in patients with T2DM and the difference reached significance in the subdomains “fatigue”, “activity” and “systemic”. It is possible to speculate that the lower CLDQ scores reported in the UK compared to Germany and Spain may be reflected by the significantly higher occurrence of T2DM in the UK (61% versus 39% and 53%) and obesity in the UK (91% versus 64% versus 59%).

In a recent review, out of 6 interventional studies treatments, only treatments targeting metabolic parameters provided evidence for QoL benefits (243) Both weight loss and liraglutide have been associated with improvement in NAFLD patient physical health with a reduction in fatigue, abdominal symptoms and health related worries. In the weight loss study, subjects who achieved a 5% reduction in weight experienced a 0.45-point improvement in the total CLDQ scores, compared to 0.003 in those who did not with nondiabetic patients with NASH and mild fibrosis more likely to do well. These studies have shown that in patients with NAFLD, significant improvements in QoL can occur that appear to be specific to weight loss and not biochemical improvements highlighting the importance of using PRO endpoint to assist the interpretation of clinical trial data (287, 301).

Fatigue and patient metabolic profile The presence of the metabolic syndrome has been closely linked to the pathogenesis of fatigue (302). In this study, BMI correlated with FIS scores (Rs 0.225, p=0.006). Sleep disruption has evolved, like metabolic disease in humans along with industrialism (303). Sleep disruption is associated with the development of diabetes in murine models and humans as in hepatocytes, peripheral clocks orchestrate many genes regulating nutrient sensing, storage and release (294, 304, 305). Sleep deprivation also affects the inflammatory response. The metabolic syndrome is characterised by low grade chronic inflammation as in NASH thus it is possible that sleep deprivation promotes steatohepatitis in NAFLD (306).

Depression and patient metabolic profile No associations between depression scores and metabolic parameters were observed. Interestingly, in a mice model of diabetes, impaired responsiveness of serotonin in response to food was observed with a resultant increase in hypothalamic serotonin secretion in diabetes thus linking diabetes, depression and NAFLD. The insulin-serotonin cross-talk is one potential critical modification in the brain during the onset of diabetes (307).

2.4.5. Relationship between depression fatigue and QoL in NAFLD

Significant correlations exist between FIS scores, depression and all aspects of the CLDQ scores. The directionality of these relationship has been questioned in publications to date (248). Regarding fatigue; patient reported fatigue may represent an emotional response to the development of chronic liver disease or perhaps, the observed QoL impairment and depression are a consequence of fatigue. These complex phenomena have been described in other chronic liver diseases and may also be applicable to NAFLD (308-311). Similar speculation can be extended to the relationship observed between fatigue and "physical activity", as to whether perceived fatigue leads to reduced physical exertion or does reduced physical activity in NAFLD lead to NAFLD and associated fatigue. Unfortunately, due to the cross-sectional design of this study, it was not possible to derive robust conclusions about cause and effect (280).

Nonetheless, based on this analysis, it can be speculated that improvement in steatohepatitis, in particular lobular inflammation and ballooning should have a measurable effect on HRQL and mood perhaps even independently of fibrosis as has been observed in QoL scores studies in HCV undergoing viral eradication therapies (312, 313).

2.4.6. Study strengths and weaknesses

Assessment tools Validated PRO measures were utilised in this study as opposed to asking open ended questions pertaining to QoL. This ensured that the questions and response options were standardised. The setting of administration i.e. in liver clinic was kept consistent. This is important as research has shown slight response differences according to administration setting (314). A 100% response rate was achieved in this study. This is important as high levels of missing PRO data can reduce study power, enhance type II error risk and potentially introduce bias (315). The collection of Patient Reported Outcomes (PROs) employed in this study were patient reported as opposed to physician reported. The consequent reduction in subjectivity was accepted in order to obtain more valuable patient-derived assessments. ***Depression scales*** the study period was over 1 year. The responses (particular mood related scales) may be subject to seasonal variation. ***Fatigue scales*** No objective measures of fatigue were used in this study (for example actigraphy) therefore any findings will need to be investigated as variables in future studies. ***CLDQ scores*** the CLDQ,

while representing a disease specific tool capable of detecting disease specific aspects that may be overlooked by generic QoL assessments (such as the SF-36), a comparison with the general population while preformed in this study is not a true comparison (316).

Study population the study was conducted in a large cohort of patients with biopsy proven NAFLD. This reduced the variability in the sub-populations studied as this cohort's histology was read by expert histopathologists with high documented kappa value for inter-observer variability in readings. However, the majority of patients enrolled in this study had not progressed to cirrhosis and none had progressed to liver failure. This may affect the generalisability of the results. This is important as there is now evidence that NASH is the second leading indication for OLT in the US and is the second most common aetiology of HCC leading to OLT (317-319). When interpreting the results of this study, it is important to note that subjects were recruited from a tertiary referral centre and not from the general population. Referral bias resulting from a population of patients with more advanced NAFLD are a truer reflection of more severe NAFLD cases rather than the largely asymptomatic cohort in the general community (243).

Missing factors Other factors have been demonstrated to affect QoL scores including levels of income, level of education and relationship status which were not assessed in this study (320, 321). The number of medications (a surrogate marker of disease morbidity) has been shown to correlate with most domains of SF-36 was not recorded (322). The development of HCC has also seen to impact QoL and was not considered in the cirrhotic cohort of this study. This is important information to capture in future studies as there is evidence to suggest that better HRQL at baseline is associated with longer survival in HCC (323). QoL in NAFLD can also be captured through qualitative concept elicitation research in "focus groups" which was not explored in this study (240).

2.4.7. Future Directions

Need for standardised PRO administration practices; Evidence from a review of 75 trials with PRO measurements included as endpoints found that only 8% had a protocol for PRO assessment (324). In the future, ***PRO analysis*** can be standardised in line with the "Setting International Standards in Analysing Patient Reported Outcomes and Quality of Life Endpoints Data" (SISAQOL) developed by international experts (325). ***PRO reporting*** can be

standardised according to the CONSORT-PRO Extension, published by the International Society for Quality of Life Research (ISOQOL)(326). ***PRO publication*** can be standardised according to CONSORT-PRO and ISOQOL guidelines (327, 328). This will serve to improve PRO data quality.

Employment of the validated NAFLD specific CLDQ The standard CLDQ was used in this study. This questionnaire version has been criticised for having shown significant differences pertaining to gender and may have a higher sensitivity in women compared to men. Cognisant of such potential shortcomings, Younossi et al recently developed the CLDQ-NAFLD for efficacy trials in NAFLD offering improved discrimination for the presence of obesity and depression (238, 329).

PRO data awaited There is a paucity of QoL data in interventional NASH trials. However, numerous phase III clinical trial, (RESOLVE-IT , REGENERATE, STELLAR 3/4) all utilise CLDQ-NASH. Data outcomes are imminently anticipated and will provide a model for PRO inclusion in future clinical trials. Ultimately, longitudinal studies to measure the evolution of the QoL burden in NAFLD from milestones such as initial diagnosis to first clinical event.

2.5. CONCLUSION

NAFLD patients suffer from significant impairment in quality of life, particularly in relation to fatigue, while their mental health appears to be less significantly affected. A variety of demographic, clinical states and biological factors have been investigated as causative agents however, the most significant contributions to impaired QoL in NAFLD appears to be from the presence of fatigue. There is a need for large, prospective longitudinal studies powered to delineate QoL correlates and summarise NAFLD patient QoL profiles in parallel to ongoing interventional RCTS in NAFLD. Delineation of the factors which drive impaired QoL in NAFLD will permit the development of therapeutic targets and increased awareness of QoL in NAFLD will allow clinicians to consider both clinical and patient factors in treatment selection (246).

CHAPTER 3.

COST OF ILLNESS STUDY ASSOCIATED WITH THE PREVALENCE, SEVERITY AND PATTERNS OF CLINICAL PRACTICE IN OUTPATIENT VISITS FOR NAFLD – THE UNITED KINGDOM CONSTANS STUDY

3.1. INTRODUCTION

3.1.1. Burden of chronic liver disease on health care resources

Liver disease mortality rates have increased 400% since 1970 with a high disease burden caused by alcohol misuse and obesity (108). Alcohol misuse cost the NHS approx. 3.5 billion per year with a loss of productivity of 7.3 billion. Obesity costs the NHS 6.1 billion per year with a loss of productivity of 5.6 billion over 2 years (109). This is reflective of a global trend with a recent US study reporting a doubling in the number of NAFLD patient referrals from 3585 to 6646 over a 5-year period paralleled by an annual cost increase attributable to the increase in the number of OPD visits and the increasing frequency of co-morbidities in this population (111). Cross-sectional UK data to corroborate these findings is lacking. Currently, the Lancet commission in the UK require such 'real time' data to justify an increase in the provision of medical and nursing training in hepatology to cope with the ever-increasing service demands (113).

3.1.2. Rationale to assess the economic burden of both NAFLD and ALD out-patient utilization

The primary objective of this study was to provide a descriptive analysis of the prevalence, severity and patterns of clinical practice in outpatient visits for NAFLD in addition to a cost-of-illness study to estimate direct medical costs from a UK perspective. A comparator group of ALD was selected as together, ALD and NAFLD constitute the majority of chronic liver disease (CLD) worldwide and are consuming an increasing proportion of healthcare resources (330). Both conditions share many pathophysiological processes and have similar histological features (201). There is a body of evidence to suggest that each aetiology of liver disease may be more correctly considered as an outcome of a multifactorial process. For example, alcohol toxicity is doubled in the setting of a BMI >35kg/m^2 (331) and in Europe, both alcohol misuse and obesity remain a significant health burden (332-334). The association between alcohol consumption and obesity is complex. Several epidemiological studies support the view that there is a strong causal relationship between consumption of a diet high in fat (and/or presence of T2DM), the consumption of alcohol and progressive liver disease (335-344). In clinical practice, in a patient with a fatty liver, it can be difficult to determine the relative contributions of alcohol consumption and the metabolic syndrome

when both risk factors are present and among patients referred to a liver clinic with a diagnosis of NAFLD, their alcohol consumption is often significantly higher than initially recognised (345). Data from the German Study of Health in Pomerania (SHIP) established the prevalence of people meeting the criteria for both NAFLD and ALD at 17.5% (346) showing that high alcohol consumption and overweight/obesity are frequently encountered in day-to-day clinical practice (42).

3.1.3. Cost-of-illness studies: explanation of concept

Jefferson et al. described a cost of illness (COI) study as an analysis "to itemize, value, and sum the costs of a particular health problem with the aim of giving an idea of its economic burden"(347). Cost of illness (COI) studies utilise data on disease incidence and prevalence and disease related direct and indirect expenditure. The costing data is a useful metric to prioritise resource allocation in health policy forums and cost estimate trends can be helpful to establish true monetary and opportunity costs associated with health care interventions. This is especially relevant to NAFLD, as while no pharmacological therapies are approved at present, there is a rapidly expanding formulary of anti-fibrotic drugs imminently due to complete Phase III clinical trials and will come into clinical use. The NAFLD drugs in trial at present, unlike for example hepatitis C drugs, involve oral regimens and have good tolerability therefore cost may be the most significant barrier to implementation. It will be important to be able to assess the cost-effectiveness of anti-fibrotic agents therefore performing a detailed real world synopsis of direct medical costs in an "anti-fibrotic treatment naïve" NAFLD population will be valuable.

3.1.3.1. Types of costs

There are three types of costs to consider (1) direct, (2) indirect (productivity losses due to morbidity and mortality), and (3) intangible costs. In this study, societal costs were not considered. The focus was to concentrate on direct medical costs with the caveat that such study subtypes often have restricted generalisability outside the health care system they were derived in. However, the goal was to present the cost analysis study in such descriptive detail as to enable other institutes to apply unit costs specific to their practice and calculate total costs for their units based on the service utilisation that was quantified in this study.

Challenges unique to NAFLD cost analysis relate to the fact that it is associated with significant co-morbidities, it has a long asymptomatic latency period and higher costs are associated with more advanced disease (108). It is also worth noting that chronic diseases (such as NAFLD) have higher direct health costs than those associated with acute or communicable diseases (348).

3.1.4. Approaches to COI studies

3.1.4.1. Prevalence- vs. incidence-based approach

A prevalence based approach estimates the financial burden of a disease over a pre-defined time period. The specified period is usually one year therefore a 12-month period+/-2 weeks was selected for this study to capture patients just falling outside the 12-month return period which may have escaped data collection otherwise. This method is in contrast to an incidence-based approach which estimates the lifetime costs of a disease (from diagnosis until cure/death) (348). Prevalence-based studies measure the volume of health outcomes attributable to diseases in a year and then calculates the resultant costs that incur as a result. This approach is the most accurate way to cost chronic conditions such as NAFLD (347). A potential disadvantage to the prevalence based method, is that unlike the incidence based approach it does not capture the long-term consequences of the conditions (349). However, the prominent health economist, Rosanna Tarricone recommended that the prevalence approach should be employed if the main study objective is to highlight a previously underestimated disease burden to provide a snapshot of the global burden of a disease and the most significant cost components (350, 351). Over the last decade, there has been speculation concerning global underestimation of the prevalence of NAFLD (6). A rigorous follow-up systemic review and meta-analytic approach of 45 studies defining NAFLD radiologically, now estimates the global prevalence of NAFLD at 25% (8). However, the previous pooled regional prevalence of NAFLD using blood tests was 12.89% for the USA and 13% for Europe. The prevalence of NAFLD is also higher in specific groups for example type II diabetics (57.8%) and the morbidly obese (80%) (352, 353). In contrast to the extensive prevalence data available on NAFLD, incidence data is infrequent with the only robust data reported from Asia and Israel estimated at 52.34 and 28.01 per 1000 person-years respectively (8). As a comparator group, ALD is also suited to a prevalence based

approach. Although the prevalence of ALD in the US is estimated to be stable and between 2-2.5%, detailed alcohol histories are not comprehensively documented in hepatology referrals and performed in less than 50% of hospital inpatients and less than 25% of primary care patients (354-356).

3.1.4.2. Prospective vs. retrospective approach

In a COI study, data collection can either be prospective or retrospective. A prospective approach involves data collection being performed in real time(351). However, considering the chronicity of NAFLD as a disease, the retrospective approach is more economical and time efficient. Pre-selected diagnostic and monitoring events have already occurred and can be immediately recorded in a dataset (351). This approach is beneficial in NAFLD as the majority of patients have mild disease therefore the bulk of management is provided in an ambulatory setting where ancillary services are requested in the study hospital generating a robust online observational dataset.

3.1.4.3. Top-down vs. bottom-up approach

There are 3 approaches to COI studies "top-down" versus "bottom-up" versus econometric. An econometric approach estimates costs based on the cost difference between the diseased cohort and a demographically matched disease free cohort. This was not suited to the analysis performed as if the mean difference approach was adopted, the data generated would be the "per case" cost of the disease rather than total cost which is less generalizable. Furthermore, a cohort matched for metabolic risk factors in the absence of hepatic steatosis would be challenging to populate. Morgenstern et al developed the "top-down" (attributable risk approach). This approach calculates attributable costs (by summating data collected with a population-attributable fraction (PAF)) to cost the proportion of a disease that is due disease/risk factor exposure (357-359). This type of analysis allows one to control for confounding variables which otherwise may cause a bias in the relative risk and consequently the PAF value. Investigators must also assess for and if present account for an "omitted third variable" by looking for the "effect measure modification" determined by the level of collinearity between variables (360, 361). This approach was not adopted in this study as it requires not only cost data but additional data on relative risks to calculate PAFs

(348). The method selected was the bottom-up approach where the estimation of costs can be divided into two steps. Step one involves quantification of health inputs. Step two involves derivation of unit costs of the inputs used with an overall estimate achieved by multiplication of unit cost by quantity. The production of a "bottom-up" perspective to NAFLD care is valuable as it is often deficient from cost estimates derived from large healthcare insurance databases from which the majority of costing data is derived (351). This approach requires data on both the unit costs of services and the frequency of use. This descriptive lay-out although exacting in collection, will enable different institutes to apply their hospital specific unit costs to the service utilisation frequencies observed in this NAFLD study.

3.1.5. Perspectives of COI Studies

COI studies can be conducted from a number of perspectives, with a different combination of cost items. Each perspective is powered to describe costs to society, the health care system, third-party payers, business, the government and participants and their families. Each perspective includes a subset of costs unique to that group from the categories of medical costs, morbidity costs, mortality costs, transportation costs and transfer payments (348). The perspective chosen can have a large effect on the actual cost estimates therefore it is important to emphasize that this NAFLD study is concerned with the medical costs of NAFLD only (362) therefore the 'Health care system' perspective was appropriate. However, the 'societal' perspective is championed as the most comprehensive and is recommended by Gold et al as the "gold standard" for all subsequent cost analyses including cost-benefit analysis and cost effectiveness analysis (363). The data requirements for the societal approach are sizable involving medical, morbidity, mortality and transportation costs. Societal costs are not considered in this study as it concentrates on direct medical costs from the perspective of the payer in an anti-fibrotic treatment naïve NAFLD population. With both the health care system and societal perspectives, higher cost estimates are expected owning to the larger range of costs associated with these perspectives (348).

3.1.6. Study Objectives

Care provision for patients with NAFLD patients is largely conducted in an ambulatory setting.

- The first objective of this study will be to define a 'cross-sectional window', comprising a 4-week period to characterise a typical OPD encounter. This will involve summarising patient profiles, the various categories of new patient referrals and the spectrum of clinical investigations 'activated' in an isolated OPD encounter. Patients with both 'known' and 'suspected' NAFLD and ALD will be identified for further follow-up.
- The second objective, will be to follow-up this cohort over the subsequent 11 months and conduct a bottom-up micro-costing study, with the set of ALD patients acting as a comparator group. Projected annual costs will be based on the assumption that a similar patient cohort will be recruited monthly.

Patient groups compared will include NAFLD versus ALD, NAFLD cirrhotics versus ALD cirrhotics and known NAFLD cases versus suspected NAFLD cases. The sample of patients recorded will involve patients along the full spectrum of NAFLD related liver disease and thus will be representative of clinical care in the UK, which is funded and delivered through the National Health Service (NHS) public health service.

3.2. MATERIALS AND METHODS

3.2.1. Patient characteristics

The flow of patients through the study is illustrated in ***Figure 3.1***. Patient recruitment, characterisation and inclusion and exclusion criteria are described in chapter 3. The flow of patients through the study is illustrated in ***Figure 3.1***. Out-patient hepatology visits at the Freeman hospital, Newcastle were recorded over a 4-week period. Patients were eligible for inclusion if they were attending for liver-related issues and were subsequently reviewed by one of seven consultant hepatologists/registrars. Liver related issues were categorised as follows; Alcoholic cirrhosis or Alcohol related disease (ALD); chronic viral hepatitis; suspected gastrointestinal cancer or cholangiocarcinoma; known non-alcoholic liver disease (NAFLD); suspected NAFLD and miscellaneous hepatic pathologies. Patients were recorded as a 'new patient' if this was the initial consultation or 'follow-up' if patients had been reviewed previously. Patients were eligible for follow-up if they met the following inclusion criteria; patients seen in out-patients for suspicion of NAFLD (steatosis or metabolic risk factors); abnormal LFTs (transaminases or GGT); high serum ferritin or cryptogenic cirrhosis. In the NAFLD arm, patients were excluded as per exclusion criteria outlined in section 2.2. For the control ALD cohort, participants had a history of or current DSM-IV diagnosis of alcohol use disorder; evidence of alcoholic liver disease (ALD) based on a thorough history, physical examination, and laboratory tests (i.e. the de ritis ratio ~2:1), which is associated with ALD or ALD cirrhosis (364).

3.2.2. Data collection and follow-up period

All outpatient visits at the hepatology clinic in the Freeman hospital were screened over a 4-week period in October 2017. This comprised the screening population. Patients with a known or suspect diagnosis of NAFLD as per pre-defined screening criteria were selected. This patient subset was then profiled in terms of reason for referral and the investigations requested as a result of this single patient encounter. This provided a 'cross-sectional snapshot' of the average general hepatology out-patient encounter. This index NAFLD cohort was then subject to a comprehensive 1 year follow-up study, where the investigations ordered, medical manpower employed and final disease stage was documented. The follow-up format involved documentation of a final diagnosis. Diagnostic

categories included NAFLD without alcohol; NAFLD with moderate alcohol; NAFLD Cirrhosis and other. This cohort represents the UK arm of the pan-European EPoS CONSTANS study. Data collected reflects current patterns of practice and was used to calculate direct medical costs incurred. In parallel to this data collection, 94 patients with ALD were selected for follow-up as a comparator cohort, with 79 patients being eligible for inclusion.

3.2.3. Micro-costing of out-patient visits

NAFLD and ALD cohorts selected from patients reviewed over a 1-month period, the "screening cohort" were followed up as appropriate over the next 11 months. ***Medical staff utilisation*** in hepatology out-patient clinic was defined as follows; 15 minutes for a review patients and 30 minutes for a new patient consultation. The unit cost associated with this time was calculated according to cost per hour as per the NHS salary scales for registrars and consultants. It was assumed that consultants reviewed two-thirds of patients and registrars one third. ***Investigations*** The ordering frequencies of standard 'liver' investigations were determined from hospital electronic records for the time period spanning from initial consultation, including all consultations up to and including October 2018. The unit costs applied to these investigations were obtained from laboratory and radiology costs supplied by the Research and Development department associated with the hospital. ***Calculation of projected annual costs*** It was assumed total costs generated from a cohort of patients over a 4-week period would be similar in subsequent months, therefore the costs derived over the 4-week study period were multiplied by 12 to obtain total costs for 1 year. In order to establish the spectrum of disease, patients were stratified into health-states according to clinical, radiological and histological criteria. This is consistent with the natural history of NAFLD and ALD. The health-states established were: mild fibrosis (F1/F2), moderate (F3) and cirrhosis (F4). The hospital electronic patient records (EPR) were interrogated to establish how many annual consultations each patient had from October 2017 to October 2018. In addition to the ambulatory care costs, patients with more advanced liver disease had significant inpatient costs associated with their care. The cost charged by the hospital for an overnight admission was added to their out-patient costs, investigations performed as an inpatient were costed as per the out-patient micro-costing

study template. This produced an annual cost of ambulatory care for patients with differing levels of NAFLD and ALD disease severity.

Investigations external to our pre-set target investigations were not costed. For example, liver transplant was not costed if it appeared as the liver transplantation service in the UK is not enrolled in Euro transplant or other international organ matching procedures so costs associated with such procedures are not included in the analysis. ***Estate costs*** were calculated as clinic room cost per hour (local hospital tariffs). Administration fees were assumed as secretary time, 6 min per patient review, costed per hour as per NHS salary scale. ***Dieticians*** were costed per hour of service (1 hour per new consultation per patient) as per NHS salary scale.

3.2.4. Ethics

This study received approval from the Ethics Committee under the remit of EPoS (Elucidating pathways of steatohepatitis).

3.2.5. Statistical analysis

Descriptive statistics including count (and percentages), mean (and standard deviation) were used to examine the aetiology and severity of liver disease in patients presenting to a tertiary hospital hepatology clinic and to describe the target investigations accessed. Continuous variables were compared using the t test and categorical variables using Chi-square test. A p-value of less than 0.05 was considered significant. Investigations were costed and multiplied by their ordering frequencies to obtain total costs incurred over a one month period. To obtain total annual costs, this figure was multiplied by a factor of 12. To investigate associations between patient characteristics at the index appointment and the total number of hepatology clinic appointments within the 12-month period, a Poisson regression model was used. In the NAFLD cohort, the total cost per patient was calculated and a multivariable regression models was generated in which the dependent variable was total cost per patient. Independent (explanatory) variables were selected in order to obtain estimates specific for the NAFLD group. Regression coefficient estimates, z and p values were reported for the model. All statistical analyses were performed using SPSS software version 24.0 (SPSS Inc, Chicago, US)

3.3. RESULTS

3.3.1. Cross sectional snapshot of patients presenting to Hepatology Outpatients Department

In the predefined 4-week window, 664 patients attended general hepatology outpatient appointments in the Freeman Hospital, Newcastle Upon Tyne. 159 patients presented for review of known or suspected NAFLD. 145 patients in this cohort were eligible for follow-up. 30 patients out of a potential 664 attendees failed to present for follow-up. 5% of this non-attending subgroup (n=6) were referred on the suspicion of NAFLD. A detailed summary characterising out-patient attendance is displayed in ***figure 3.1.*** The mean age of the screening cohort was 58 +/- 16 years.

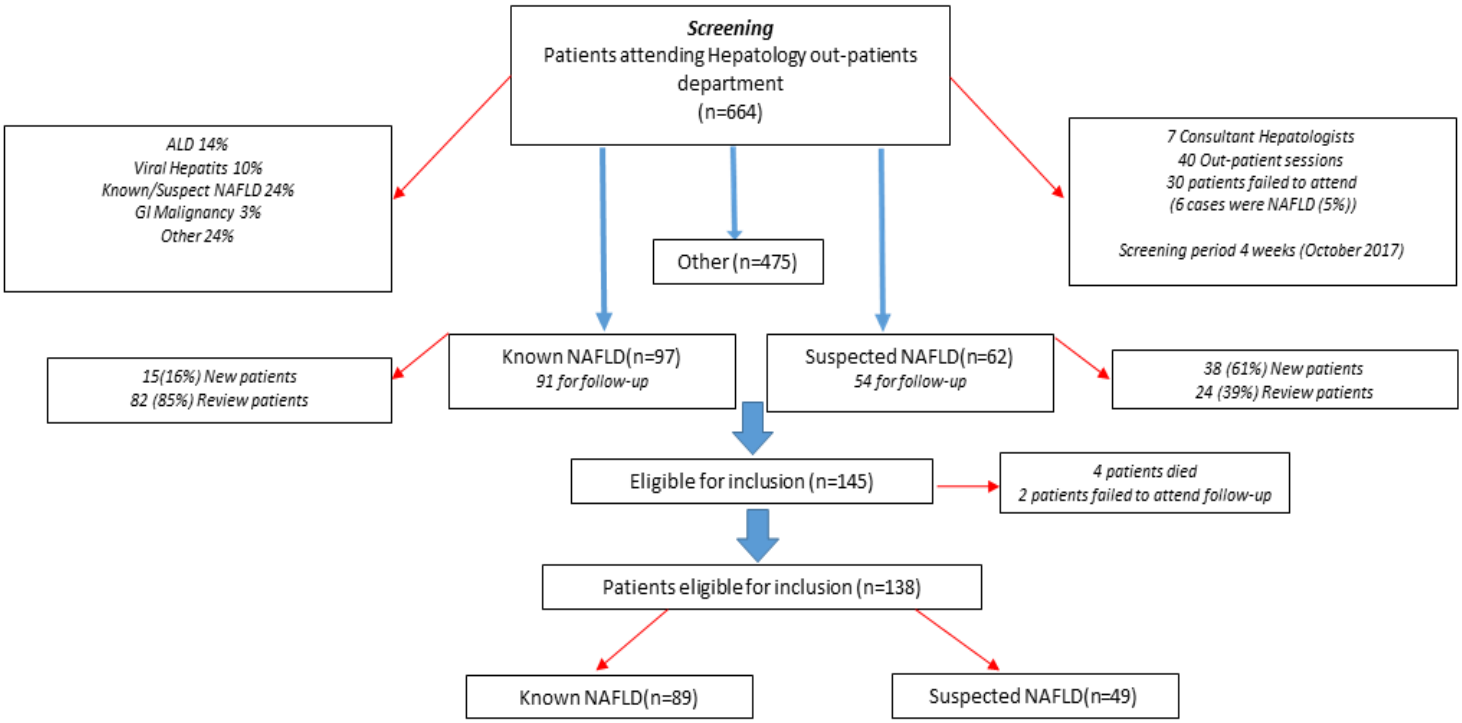


Figure 3.1: Work Flow of Patients

3.3.2. Reason for Consultation

The majority of patients attending for general hepatology OPD review presented with a diagnosis of known/suspected NAFLD (24%). Other aetiologies included alcoholic liver disease (ALD) (14%), orthotopic liver transplant (OLT) review (12%), primary biliary cirrhosis (PBC) (11%) and chronic viral hepatitis (9%). A detailed breakdown of the liver disease aetiologies presenting to the general hepatology OPD is presented in ***table 3.1.***

Table 3.1: Reason for consultation in the screening Cohort (n=664)

Mean age	58 +/- 15.6 years (range 17-96)		
Alcoholic Liver Disease	91	14%	
Chronic Viral Hepatitis	63	9%	
Suspected NAFLD	62	9%	
Altered Liver Function Tests	28	4%	
Cryptogenic Cirrhosis	9	1%	Combined
Hyperferritinaemia	5	1%	NAFLD 24%
Steatosis on Imaging	20	3%	
Known NAFLD	97	15%	
Suspected Gastrointestinal Malignancy	23	3%	
Other	328	49%	
Autoimmune Hepatitis	61	9%	
Haemachromatosis	32	5%	
Orthotopic Liver Transplant follow-up	81	12%	
Primary Biliary Cirrhosis	73	11%	
Miscellaneous Hepatic Pathologies	81	12%	

3.3.3.

3.3.3. Follow-up visits and services requested

145 patients were eligible for follow up from the known (n=97) and suspect (n=62) NAFLD patient cohorts presenting to the general hepatology OPD. In the follow-up NAFLD cohort, 3 patients died. One death was associated with advanced age (89 years), the second with alcohol excess in the context of dual aetiology fatty liver disease and the third succumbed to sepsis associated with immunosuppressive therapy. A further 4 patients failed to attend for follow-up with a resultant 138 patients in the final follow-up cohort. ***(Figure 3.1)*** The investigations requested at the initial consultation are summarised in ***table 3.2*** and are reflective of the spectrum of investigations 'activated' in a typical OPD encounter. Both cohorts had a similar time period to next OPD follow-up

Table 3.2: Spectrum of service utilisation requested at initial patient interaction in NAFLD

Total cohort (n=159)	**Known NAFLD (n=97)**	**Suspected NAFLD (n=62)**
Mean age	62.8+/-11.9 years	58.5 +/- 17.6 years
Blood tests	91 (93.8%)	54 (87.1%)
Imaging	45 (46.4%)	17 (27.4%)
Ultrasound	39 (87%)	16 (94%)
CT Scan	5 (11%)	
MRI Scan	1 (2%)	1 (6%)
Fibroscan	12 (12.4%)	23 (37.1%)
Liver Biopsy	4 (4.1%)	1 (1.6%)
New Patient	15 (15.5%)	38 (61.3%)
Review Patient	82 (84.5%)	24 (38.7%)
Eligible for follow-up	89 (92%)	49 (79%)
Time to first OPD follow-up	215 +/- 103 days	204 +/- 115 days
Number of OPD follow-ups	2.3	2.4

62 patients were reviewed for suspected NAFLD and 54 were offered a follow-up review appointment. In total 49 patients returned for follow-up and were eligible for inclusion. A summary of patient age and metabolic profile in each of the two study groups are recorded in ***table 3.3.***

Table 3.3: Follow-up Cohort, Metabolic Profile			
	Total NAFLD (n=138)	**Known NAFLD (n=89)**	**Suspected NAFLD (n=49)**
Mean Age	60.6 +/-14.4 years	63 +/- 11.9 years	56.6+/- 17.7 years
Metabolic Parameters			
BMI	34.3 +/- 9.3	35.1+/-8.4	32.9 +/- 10.9
Type 2 Diabetes	66 (47.8%)	51 (57.3%)	15 (30.6%)
Hypertension	76 (55.1%)	54 (60.7%)	22 (44.9%)
Dyslipidaemia	77 (55.8%)	61 (68.5%)	16 (32.7%)
Obstructive Sleep Apnoea	9 (6.5%)	7 (8%)	2 (4.1%)

3.3.4. Patient Profile "Suspect NAFLD" patient cohort

Table 3.4 summaries the 'reason for referral' categories recorded in the suspected NAFLD cohort. Altered liver function tests were the most common reason for referral, documented in 41% of cases.

Table 3.4: Follow-up Cohort, suspected NAFLD (n=49), Reason for consult	
Suspected NAFLD (n=49)	
Time to First OPD follow-up	204 +/- 115 days
Number of OPD	2.4
Reason for referral	
Altered Liver function tests	20 (40.8%)
Cryptogenic Cirrhosis	7 (14.3%)
Hyperferritinaemia	3 (6.1%)
Steatosis on Imaging	19 (38.8%)

The investigations requested at the initial screening OPD appointment in patients with suspect NAFLD, stratified by reason for referral is shown in ***table 3.5****.* The most common serological investigation requested was liver function tests documented in 98% of cases. Fibroscan™ was the most common fibrosis staging investigation requested in 51% of the cohort while USS was the most common imaging modality requested in 39% of cases.

Table 3.5: Services requested at first general hepatology OPD as per reason for consult					
	Suspected NAFLD Cohort (n=49)	**Altered Liver function tests (n=20)**	**Cryptogenic Cirrhosis (n=7)**	**Hyper-ferritinaemia (n=3)**	**Steatosis on Imaging (n=19)**
Ultrasound	19 (38.8%)	9 (45%)	3 (42.9%)	1 (33.3%)	6 (31.8%)
CT scan	3 (6.1%)	2 (10%)	1 (14.3%)	0	0
MRI	4 (8.2%)	3 (15%)	1 (14.3%)	0	0
Liver Function tests	48 (98%)	20 (100%)	6 (85.7%)	3 (100%)	19 (100%)
Liver Screen	25 (51%)	9 (45%)	3 (42.9%)	1 (33.3%)	12 (63.2%)
Lipid Profile	32 (65.3%)	12 (60%)	3 (42.9%)	2 (66.7%)	15 (78.9%)
Glucose	27 (55.1%)	11 (55%)	3 (42.9%)	1 (33.3%)	12 (63.2%)
Fibroscan	25 (51%)	7 (35%)	1 (14.3%)	2 (66.7%)	15 (78.9%)
Liver Biopsy	6 (12.2%)	2 (10%)	1 (14.3%)	0	3 (15.8%)
Dietician	6 (12.2%)	0	0	0	6 (31.6%)
Hospitalisation	6 (12.2%)	1 (5%)	3 (42.9%)	0	2 (10.5%)

In the suspect NAFLD cohort (n=49), NAFLD was confirmed in 40 cases (82%). 9 patients had alternative diagnoses. ***Table 3.6.*** summarises the conclusive diagnosis and liver disease stage in this group. The spectrum of disease severity is shown in ***table 3.6*** and is stratified as follows; (F0/F1/F2/F3/F4/undetermined) -(3/0/1/3/11/31) respectively. Liver biopsy was performed in 6 patients over the study period (12%). With regard to undetermined cases (n=31), a fibroscan was performed in 55% of cases (n=17). The mean fibroscan reading was <8kPa consistent with a low probability of having moderate/advanced fibrosis.

Table 3.6: Diagnosis and Stage of Liver Disease, suspected NAFLD Cohort	
Suspected NAFLD Cohort (n=49)	
Confirmed NAFLD	40 (82%)
NASH Cirrhosis	6 (12.2%)
NAFLD	22 (44.9%)
NAFLD and Alcohol	12 (24.5%)
Other	9 (12%)
Autoimmune Hepatitis	1 (1%)
Benign Recurrent Intrahepatic cholestasis	1 (1%)
Drug induced liver injury	1 (1%)
Lymphoma	1 (1%)
Primary sclerosing cholangitis	1 (1%)
Liver Sarcoid	1 (1%)
Cholestatic LFTS	1 (1%)
Inflammatory bowel disease related liver disease	2 (2%)
De novo Liver Biopsy	6 (12.2%)
Diagnosis (n=6)	
NASH Cirrhosis	1 (17%)
NAFLD	3 (50%)
NAFLD and Alcohol	1 (17%)
Other (Autoimmune Hepatitis)	1 (17%)
Histological Liver Disease Stage	
F0	3 (6.1%)
F1	0
F2	1 (2%)
F3	3 (6.1%)
F4	11 (22.4%)
Undetermined	31 (63.4%)
Undetermined Category (n=31)	
Fibroscan Reading	17 (55%)
Mean reading	7.46KPa +/- 4.0

A summary of the conclusive diagnoses based on reason for referral is provided in ***Table 3.7***. Altered liver function tests appear to have the lowest discriminating power for NAFLD.

Table 3.7: Diagnosis in suspected NAFLD Cohort, based on reason for consult

	Total Cohort (n=49)	Altered Liver function tests (n=20)	Cryptogenic Cirrhosis (n=7)	Hyper-ferritinaemia (n=3)	Steatosis on Imaging (n=19)
NASH Cirrhosis	6 (12.2%)	1 (5%)	3 (42.9%)		2 (11%)
NAFLD	22 (44.9%)	4 (20%)	1 (14.3%)	3 (100%)	14 (73.5%)
NAFLD and Alcohol	12 (24.5%)	6 (30%)	3 (42.9%)		3 (15.5%)
Other	9 (12%)				
AIH	1 (1%)	1 (5%)			
BRIC	1 (1%)	1 (5%)			
DILI	1 (1%)	1 (5%)			
Lymphoma	1 (1%)	1 (5%)			
PSC	1 (1%)	1 (5%)			
Liver Sarcoid	1 (1%)	1 (5%)			
Cholestatic LFTS	1 (1%)	1 (5%)			
IBD Related Liver Disease	2 (2%)	2 (10%)			

AIH=Autoimmune Hepatitis, BRIC=Benign Recurrent Intrahepatic cholestasis, DILI=Drug induced liver injury, PSC=Primary sclerosing cholangitis, LFTs= Liver function tests, IBD= Inflammatory bowel disease

3.3.5. Patient Profile, "Known NAFLD" cohort

The investigations requested at the initial screening OPD appointment in patients with known NAFLD are shown in ***table 3.8***. Hospitalisation episodes had the highest recorded frequency in the known NAFLD cohort. Liver biopsy request frequencies are higher in the suspected NAFLD cohort.

Table 3.8: Service utilisation arising from screening visit one in NAFLD Cohorts

	Suspected NAFLD Cohort (n=49)	Known NAFLD Cohort (n=89)	Total NAFLD Cohort (n=138)
Ultrasound	19 (38.8%)	49 (55%)	68 (49.3%)
CT scan	3 (6.1%)	11(12.4%)	14 (10.1%)
MRI	4 (8.2%)	6 (6.7%)	10 (7.2%)
Liver Function tests	48 (98%)	88 (98.9%)	136 (98.6%)
Liver Screen	25 (51%)	13 (14.6%)	38 (27.5%)
Lipids	32 (65.3%)	66 (74.2%)	98 (71%)
Glucose	27 (55.1%)	44 (49.4%)	71 (51.4%)
Fibroscan	25 (51%)	25 (28%)	50 (36.2%)
Liver Biopsy	6 (12.2%)	3 (3.4%)	9 (6.5%)
Dietician	6 (12.2%)	13 (14.6%)	19 (13.8%)
Hospitalisation	6 (12.2%)	27 (30.3%)	33 (23.9%)

A summary of liver disease stage in the known NAFLD group is shown in ***table 3.9***. The spectrum of disease is as follows (F0/F1/F2/F3/F4/undetermined) - (1/1/5/6/44/32) respectively. In the undetermined cases (n=32), fibroscan was performed in 15 cases (47%) where average readings approximated 8kPa indicating a low probability of having advanced fibrosis/cirrhosis.

Table 3.9: Diagnosis and Stage of Liver Disease, known NAFLD Cohort

Known NAFLD Cohort (n=89)	
Confirmed NAFLD	89 (100%)
NASH Cirrhosis	44 (49%)
NAFLD	37 (42%)
NAFLD and Alcohol	8 (9%)
De novo Liver Biopsy	3 (3.4%)
Histological Liver Disease Stage	
F0	1(1.1%)
F1	1(1.1%)
F2	5 (6%)
F3	6 (7%)
F4	44 (49.4%)
Undetermined	32 (36%)
Undetermined Category (n=32)	
Fibroscan Reading	15 (47%)
Mean reading	8.05KPa +/- 4.6

3.3.6. Trends observed in OPD appointments

When categorized by disease severity, with disease progression, there was a stepwise increase in the proportion of patients having four or more follow up appointments and a corresponding decrease in the proportion with one further appointment ***(Figure 3. 2.)***. The Poisson regression indicated that the performance of liver biopsy was significantly predictive of the number of hepatology clinic appointments attended in the following 12 months ***(Table 3.10).*** An IRR (incidence risk ratio) of 0.617 (p=0.019) indicates a lesser number of appointments will be requested if a liver biopsy is performed. One can speculate, that less follow-up is required once diagnostic certainty is disease stage is eliminated.

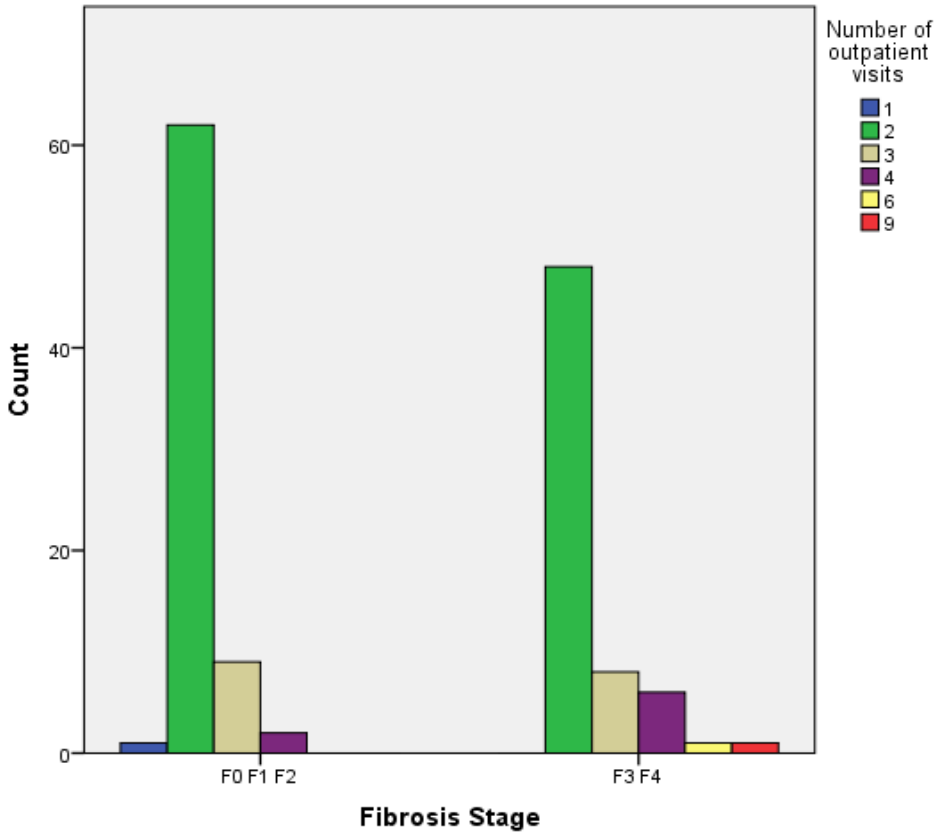


Figure 3.2: Proportion of patients with and without advanced liver disease, who had one, two, three or four, or more than four subsequent clinic appointments over the 12-month period

Table 3.10: Summary of the Poisson regression model investigating patient factors as potential predictors of number of hepatology clinic appointments (dependent variable)			
Independent variables	**IRR**	**95% Confidence intervals**	**P-value**
Patient Characteristics			
Age	0.996	0.989-1.004	0.316
BMI	0.996	0.983-1.008	0.501
Known NAFLD	1.081	0.861-1.356	0.503
Suspected NAFLD	0.925	0.737-1.161	0.503
Disease Phenotype			
NAFLD Cirrhosis	0.935	0.748-1.168	0.553
Advanced dx	0.904	0.668-1.224	0.515
Mild dx	1.106	0.817-1.485	0.515
Undetermined dx	0.995	0.790-1.254	0.967
Patient Metabolic Profile			
Diabetes	0.918	0.719-1.174	0.496
Hypertension	1.063	0.823-1.373	0.640
Dyslipidaemia	1.085	0.832-1.414	0.546
Investigations Performed			
Abdominal USS	1.014	0.807-1.274	0.904
Fibroscan	1.112	0.275-4.498	0.882
Hospitalisation	0.996	0.728-1.283	0.814
Liver Biopsy	0.617	0.412-0.924	**0.019**

3.3.7. Patient Profile, "Alcoholic liver disease" comparison cohort

A detailed summary of the ALD follow-up cohort is shown in ***figure 3.3.*** From the initial screening cohort of 664 patients attending for hepatology OPD review, 91 patients were identified for possible follow-up with 79 patients qualifying as eligible.

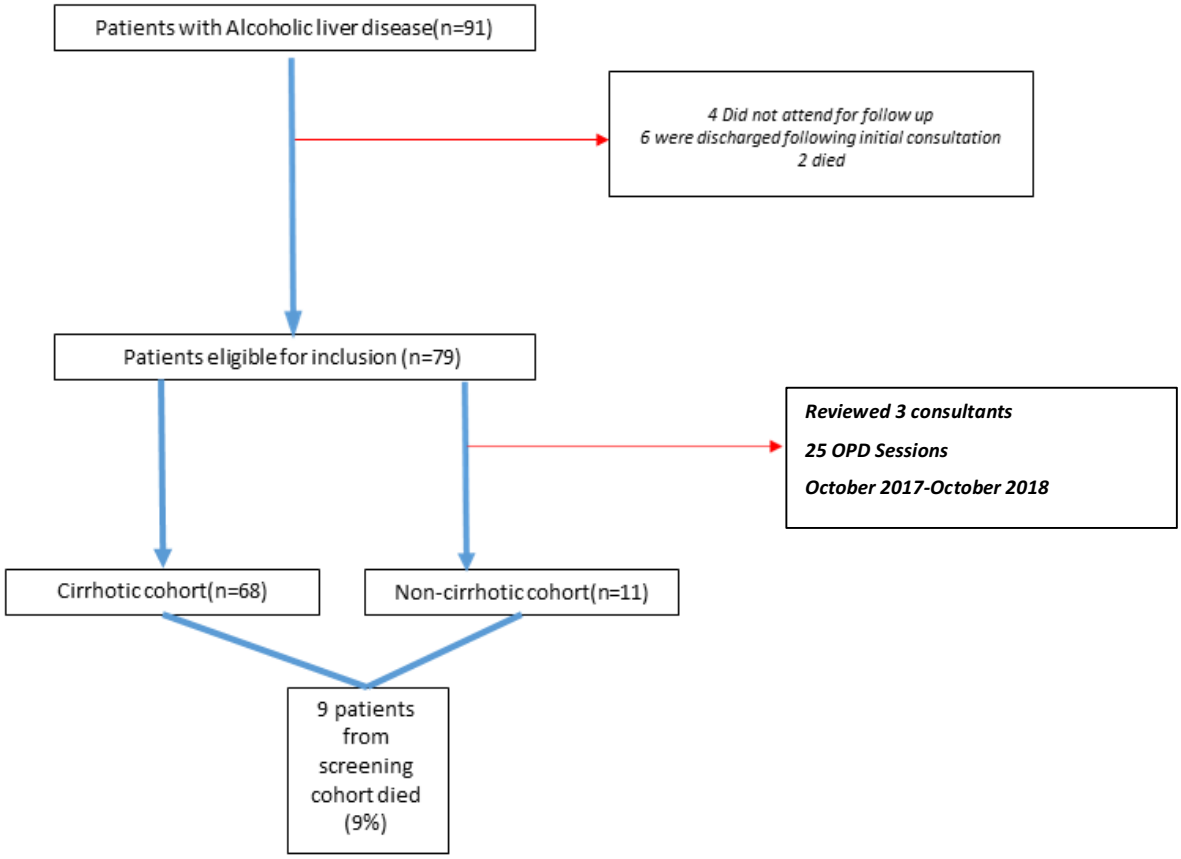


Figure 3.3: Work Flow of Patients with Alcoholic liver disease (ALD)

NAFLD and ALD cohort patient characteristics are summarised in ***table 3.11.*** The comparison groups were similar in terms of age and time to OPD follow-up. As expected, they exhibited statistically significant differences in terms of their metabolic parameters.

Table 3.11: Follow-up cohort: Alcoholic liver disease and Total NAFLD (Metabolic Profile, Out-patient utilisation)			
	Known/Suspect NAFLD (n=138)	**ALD (n=79)**	**p-value**
Mean Age	60.6 +/-14.4 years	59.5 +/- 11.3	0.564
Type 2 Diabetes	66 (47.8%)	9 (11.4%)	<0.0001
Hypertension	76 (55.1%)	12 (15.2%)	<0.0001
Dyslipidaemia	77 (55.8%)	9 (11.4%)	<0.0001
Time to first OPD follow-up	212 +/- 107	204 +/- 105	0.592
**T-test for continuous variables, Chi-square for categorical variables*			

The spectrum of investigations requested stemming from the initial patient encounter in the two cohorts are shown in ***Table 3.12***. The groups were similar in terms of the number of MRI requests, the performance of serological and staging investigations and the number of hospital days. Differences are observed between the groups in terms of disease stage and are shown in ***Table 3.13***. The majority of patients in the ALD group have advanced disease/cirrhosis while in comparison, a larger proportion of the NAFLD group have liver disease which is not staged (p<0.0001).

Table 3.12: Service utilisation summary from screening visit one in NAFLD and ALD cohort over 12 month			
	Total NAFLD Cohort (n=138)	**ALD Cohort (n=79)**	**P value**
Ultrasound	68 (49.3%)	62 (78.5%)	<0.0001
CT scan	14 (10.1%)	21 (26.6%)	<0.0001
MRI	10 (7.2%)	11 (13.9%)	0.054
Liver Function tests	136 (98.6%)	79 (100%)	0.283
Liver Screen	38 (27.5%)	10 (12.7%)	<0.0001
Lipids	98 (71%)	5 (6.3%)	<0.0001
Glucose	71 (51.4%)	5 (6.3%)	<0.0001
Fibroscan	50 (36.2%)	14 (17.7%)	0.005
Liver Biopsy	9 (6.5%)	0	0.030
Hospitalisation Incidence	33 (23.9%)	23 (29%)	0.418
Average Number of days	3 +/-4	9 +/-14	0.006
BMI<25/Abstinent Alcohol	17 (12%)	49 (62%)	<0.0001

Table 3.13: Diagnosis and Stage of Liver Disease, ALD Cohort			
	ALD (n=79)	**NAFLD (n=138)**	**P-Values**
Confirmed	79 (100%)	129 (93%)	
ALD/NAFLD Cirrhosis	68 (86%)	55 (42%)	<0.0001
ALD/NAFLD	11 (14%)	54 (42%)	<0.0001
ALD + NAFLD		20 (16%)	
De novo Liver Biopsy	0	9	0.003
Histological Liver Disease Stage			<0.0001
F0	0	4 (3%)	
F1	0	1 (1%)	
F2	1 (1%)	6 (4%)	
F3	2 (3%)	9(7%)	
F4	68 (86%)	55 (40%)	
Undetermined	8 (10%)	63 (46%)	
Undetermined Category	**N=8**	**N=59**	<0.0001
Fibroscan Reading	6 (75%)	31 (53%)	
Mean reading	11.6KPa +/- 1.3	7.8KPa +/- 4.3	
**T-test for continuous variables, Chi-square for categorical variables*			

The NAFLD and ALD cohorts, selected from the 'cross-sectional snapshot' of general hepatology OPD attendees were followed up over the subsequent 11 months. During this follow-up period, service utilization frequencies were recorded. This enabled the calculation of a total projected cost for a set volume of NAFLD and ALD patients that were followed up for one year. In line with an assumption that a similar profile of patient referrals would be generated each month, this figure for projected costs was multiplied by a factor of 12 to generate total projected annual OPD costs for ALD and NAFLD patients in the community.

3.3.8. OPD Micro-costing study: Total NAFLD versus ALD cohorts (Table 3.14)

3.3.8.1. Imaging

In both groups, the imaging modality with the highest request frequency was USS. The total number of USS requested over the 12-month period accounted for greater than 2/3 of the total cost of imaging. Imaging costs were slightly higher in the ALD group, where one can reasonably speculate that this cost difference was due to the higher number of CT scans requested in this group.

3.3.8.2. Non-invasive assessment

The non-invasive fibrosis screening modality with the highest request frequency was Fibroscan ^{TM}. A higher proportion of fibroscan requests were made in the NAFLD cohort likely explained by the large number of new referrals and higher proportion of "undetermined" fibrosis stage cases. Less fibroscans were requested in the ALD cohort, were diagnostic uncertainty was less in a largely cirrhotic cohort.

3.3.8.3. Bloods

The total cost of serological investigations was significantly higher in the NAFLD group versus the ALD group across all areas. Standard liver function tests account for the majority of costs in both groups. Liver screen, lipid and glucose panels accounted for 24% of investigations in the NAFLD group versus 5% in the ALD group reflecting the high number of suspected NAFLD cases and need to monitor metabolic dysregulation in NAFLD. AFP accounted for 7% of investigations in the NAFLD group versus 14% in the ALD group reflecting the higher number of cirrhotic patients in the ALD cohort (86% of patients in the ALD group were cirrhotic versus 40% of patients in the NAFLD group).

3.3.8.4. Procedures

The NAFLD group had a greater number of liver biopsies performed accounting for 43% of procedures. No liver biopsies were performed in the ALD cohort owing to the fact that 86% of the cohort had a diagnosis of cirrhosis confirmed by previous biopsy or radiology. OGDs accounted for 100% of procedures performed in the ALD group likely as a result of the performance of variceal assessment/management in a largely cirrhotic cohort.

3.3.8.5. Hospitalisation

Hospitalisation costs were significantly higher in the ALD group as compared to the NAFLD group. More than double the amount of hospitalisation days were recorded in the ALD group secondary to admissions for decompensated end stage liver disease and for liver transplant work-ups in a population with more advanced disease.

3.3.8.6. Medical personnel

Total medical personnel costs at each consultation were higher in the NAFLD group due to the larger number of referrals with known or suspected NAFLD (n=138) versus the smaller number of patients in the ALD cohort (n=79). New patients are given a 30-min slot versus a 15-min slot for a review patient. Nursing review and referral to specialist services (dietician) were higher in the NAFLD group.

3.3.8.7. Facility fee/Administration

Monthly administration and facility fees were higher in the NAFLD subgroup owing to the larger number of referrals in the NAFLD group requiring longer clinical sessions with health care professionals and the resultant generation of more paperwork.

3.3.8.8. Total Annual cost

The figures populating the associated table are based on a specific volume of NAFLD and ALD patient reviewed over a 4-week period and followed-up for one year. To obtain an annual cost estimate the final costs were multiplied by a factor of 12. The projected annual costs relating to NAFLD were estimated at £935,993.28 compared to £1,309,214.16 for ALD. However, if costs associated with hospitalisation are excluded, costs are higher in the NAFLD group (£589,490.76 versus £425,823.12) most likely reflective of the larger number of NAFLD patients reviewed. The mean cost per NAFLD patient is £565.21 compared to £1,381.03 for an ALD patient.

Table 3.14. OPD Micro-costing study: Total NAFLD versus ALD cohorts

Costing Analysis: Total NAFLD versus ALD (Over a 1 month period)					
Procedure	**Unit cost**	**Total NAFLD(n=138)**	**Cost (£)**	**ALD(n=79)**	**Cost (£)**
Imaging					
US	70.62	108	7626.96	112	7909.44
CT	305.1	16	4881.6	30	9153
MRI	357.62	10	3576.2	11	3933.82
		134	**16084.76**	153	**20996.26**
Non-invasive Assessment					
Fibroscan	136.13	50	**6806.5**	14	**1905.82**
Bloods					
LFTS	12.98	315	4088.7	150	1947
Electrolyte profile	5.84	315	1839.6	150	876
FBC	5.04	315	1587.6	150	756
COAG	5.62	315	1770.3	150	843
Liver screen	91	38	3458	10	910
AFP	9.91	127	1258.57	133	1318.03
Lipid	4.98	229	1140.42	13	64.74
Glucose	3.02	174	525.48	12	36.24
		1828	**15668.67**	736	**6751.01**
Procedures					
Liver Biopsy	157.35	9	1416.15	0	0
OGD	202.25	12	2427	16	3236
		21	**3843.15**	16	**3236**
Hospitalisation					
1 day Hospital	317.31	91	**28875.21**	232	**73615.92**
Medical Personnel					
Consultant hours	77.31	33	2551.23	14	1082.34
Registrar Hours	52.62	17	894.54	7	368.34
Dietician	32.9	19	625.1	0	0
Nurse	27.9	50	1395	21	585.9
		119	**5465.87**	42	**2036.58**
Facility Fee/Admin					
Room charge per hour	20.83	50	1041.5	21	437.43
Secretary to type letters	15.27	14	213.78	8	122.16
		64	1255.28	29	559.59
Total Costs			**77999.44**		**109101.18**
Total Costs - Hospitalisation			**49124.23**		**35485.26**
Total Annual cost (X12)			**935993.28**		**1,309,214.16**
Annual cost per patient			**565.21**		**1,381.03**

3.3.9. OPD Micro-costing study - Total NAFLD versus ALD- Cirrhotics (Table 3.15)

In addition to the higher ambulatory care cost normally associated with an increased numbers of review appointments, patients with more advanced liver disease (cirrhosis) tend to have additional costs associated with inpatient admissions. In terms of disease stage, cirrhosis was disproportionately represented in the ALD cohort (86%). In the NAFLD cohort, a cohort of 55 patients with cirrhosis was identified. This pre-selected cohort (n=55) was then compared to the cirrhotic ALD cohort (n=66) to generate a more comparable 'real world' micro-costing analysis.

3.3.9.1. Imaging

Overall, 76% (102 out of a total of 134 investigations) of the imaging investigations requested in the NAFLD cohort were in cirrhotic patients. This included 75% of USS (81/108), 88% of CT (14/16) and 70% (7/10) of MRI. However, imaging costs in the ALD cirrhotic population were higher likely due to the necessity for more sophisticated imaging techniques (CT, MRI).

3.3.9.2. Non-invasive assessment

This population cohort had no ambiguity relating to conclusive disease stage. The request for non-invasive fibrosis assessments was small accounting for just <1% of total investigations requested in this cohort (n=1736).

3.3.9.3. Bloods

Serological investigations in the NAFLD cohort reflected 47% of the total tests performed (811/1736). When stratified in terms of baseline bloods, liver screens and AFP, ordering frequencies were similar between the groups. The marginal extra cost in the NAFLD cohort was largely explained by the necessity to monitor metabolic risk factors (lipid and glucose levels).

3.3.9.4. Procedures

NAFLD patients with cirrhosis accounted for 100% of total OGDs and 33% of liver biopsies requested. This was similar to the ALD cohort.

3.3.9.5. Hospitalisation

Hospitalisation costs remained significantly higher in the ALD group (£73615.92) compared to the NAFLD group (£25384.80). Within the NAFLD group, cirrhotic patients were responsible for 89% of hospitalisation days (80/90 days).

3.3.9.6. Medical personnel

Costs at medical review were similar between NASH and ALD cirrhotics. A marginally higher cost was observed in the NAFLD cohort due to the recruitment of specialist services (dietician).

3.3.9.7. Facility fee/Administration

Administration and facility fees were similar between NAFLD and ALD cirrhotic groups.

3.3.9.8. Total Annual cost

The projected annual costs associated with NAFLD cirrhotics amounted to £612,409 compared to £1,261.084.8 in ALD cohort. The mean cost per NAFLD cirrhotic patient was £927.89 compared to £1,592.28 for an ALD cirrhotic patient.

Table 3.15: OPD Micro-costing study: NAFLD cirrhotics versus ALD cirrhotics

Costing Analysis: NAFLD cirrhotics versus ALD cirrhotics					
Procedure	**Unit cost**	**NAFLD Cirrhosis (n=55)**	**Cost**	**ALD Cirrhosis (n=66)**	**Cost**
Imaging					
US	70.62	81	5720.22	99	6991.38
CT	305.1	14	4271.4	29	8847.9
MRI	357.62	7	2503.34	11	3933.82
		102	12494.96	139	19773.1
Non-invasive Assessment					
Fibroscan	136.13	10	1361.3	4	544.52
Bloods					
LFTS	12.98	130	1687.4	133	1726.34
Electrolyte profile	5.84	130	759.2	133	776.72
FBC	5.04	130	655.2	133	670.32
COAG	5.62	130	730.6	133	747.46
Liver screen	91	10	910	9	819
AFP	9.91	127	1258.57	91	901.81
Lipid	4.98	90	448.2	10	49.8
Glucose	3.02	64	193.28	11	33.22
		811	6642.45	653	5724.67
Procedures					
Liver Biopsy	157.35	3	472.05	0	0
OGD	202.25	12	2427	16	3236
		15	**2899.05**	16	**3236**
Hospitalisation					
1 day Hospital	317.31	80	**25384.8**	232	**73615.92**
Medical Personnel					
Consultant hours	77.31	11	850.41	11.8	912.258
Registrar Hours	52.62	6	315.72	6	315.72
Dietician	32.9	6	197.4	0	0
Nurse	27.9	16.5	460.35	17.8	496.62
		39.5	**1823.88**	35.6	**1724.598**
Facility Fee/Admin					
Room charge per hour	20.83	16.5	343.695	17.8	370.774
Secretary to type letters	15.27	5.5	83.985	6.6	100.782
		22	**427.68**	24.4	**471.556**
Total Costs			51034.12	1104	105090.4
Total Annual cost (X12)			612,409.44		1,261,084.8
Total Costs - Hospitalisation			25649.32	872	31474.44
Annual cost per patient			**927.89**		**1,592.28**

3.3.10. OPD Micro-costing study - Known NAFLD versus Suspected NAFLD (Table 3.16)

The incidence of NAFLD is increasing, a major contributing factor to this increase is the growth in specialist referrals being made from primary care. The NAFLD cohort was stratified into a 'Known' and 'Suspected' NAFLD groups to evaluate the costs triggered by a suspected diagnosis of NAFLD. It is noteworthy that in the suspect NAFLD cohort included for follow-up; the diagnosis was confirmed in 82% of cases, translating into ongoing medical resource utilisation either in primary or secondary care.

3.3.10.1. Imaging

79% (106/134) of the total number of imaging investigations requested in the NAFLD population were in the known NAFLD group. USS was the most popular imaging modality requested in both populations.

3.3.10.2. Non-invasive assessment

Fibroscan ^{TM} was performed in 28% of the known NAFLD population in comparison to 51% of the suspect population; reflecting an urgency to non-invasively screen for advanced disease in a newly referred population to determine the need for further specialist follow-up.

3.3.10.3. Bloods

64% of the total serological investigations requested were in the known NAFLD group. The majority of the costs incurred in the known NAFLD group were speculated to be as a result of the large volume of basic investigations requested for serial monitoring of this high-risk patient group. Not surprisingly, liver screens were requested at a higher frequency in the suspect NAFLD cohort. An increased frequency of AFP requests was also observed and anticipated in the known NAFLD cohort due to the presence of 55 patients with cirrhosis.

3.3.10.4. Procedures

A larger number of procedures were requested in the known NAFLD group, mainly comprising of OGDs. A higher number of liver biopsies were performed in the suspected NAFLD group, likely for staging/ conclusive diagnostic purposes.

3.3.10.5. Hospitalisation

Hospitalisation costs in were higher in the known NAFLD group (£26,654.04) as compared to the suspect NAFLD group (£2292.30).

3.3.10.6. Medical personnel

Medical personnel costs were similar between the known and suspect NAFLD group with slightly higher costs in the known NAFLD group reflecting the larger patient number in this group.

3.3.10.7. Facility fee/Administration

Administration and facility fees were similar between both groups with marginally higher costs in the known NAFLD group, again reflective of the larger population number.

3.3.10.8. Total Annual cost

Known NAFLD patients generated higher total projected annual costs compared to suspect NAFLD patients (£691,084.92 versus £244,908.36), which is a finding most likely reflective of the higher costs associated with the higher prevalence of advanced disease in the former group. The mean cost per suspected NAFLD patient is £200 compared to £364 for a known NAFLD patient. A figure for the average "suspected" NAFLD patient is a useful metric to have in a NAFLD costing bank considering the steady increase in the number of referrals being made in primary care annually.

Table 3.16: OPD Micro-costing study Known versus Suspected NAFLD

Costing Analysis: Known NAFLD versus Suspect NAFLD					
Procedure	**Unit cost**	**Known NAFLD(n=89)**	**Cost**	**Suspect NAFLD(n=49)**	**Cost**
Imaging					
US	70.62	86	6073.32	22	1553.64
CT	305.1	14	4271.4	2	610.2
MRI	357.62	6	2145.72	4	1430.48
		106	**12490.44**	28	**3594.32**
Non-invasive Assessment					
Fibroscan	136.13	25	**3403.25**	25	**3403.25**
Bloods					
LFTS	12.98	198	2570.04	117	1518.66
Electrolyte profile	5.84	198	1156.32	117	683.28
FBC	5.04	198	997.92	117	589.68
COAG	5.62	198	1112.76	117	657.54
Liver screen	91	13	1183	25	2275
AFP	9.91	108	1070.28	19	188.29
Lipid	4.98	147	732.06	82	408.36
Glucose	3.02	102	308.04	72	217.44
		1162	**9130.42**	666	**6538.25**
Procedures					
Liver Biopsy	157.35	3	472.05	6	944.1
OGD	202.25	9	1820.25	3	606.75
		12	**2292.3**	9	**1550.85**
Hospitalisation					
1 day Hospital	317.31	84	**26654.04**	7	**2221.17**
Medical Personnel					
Consultant hours	77.31	17	1314.27	16	1236.96
Registrar Hours	52.62	9	473.58	8	420.96
Dietician	32.9	13	427.7	6	197.4
Nurse	27.9	26	725.4	24	669.6
		65	**2940.95**	54	**2524.92**
Facility fees/Admin					
Room charge per hour	20.83	26	541.58	24	499.92
Secretary to type letters	15.27	9	137.43	5	76.35
		35	679.01	29	576.27
Total Costs		1489	**57590.41**		**20409.03**
Total Costs - Hospitalisation		1405	**30936.37**		**18187.86**
Total Annual cost (X12)			**691084.92**		**244908.36**
Cost per patient per year			£364.52		£200.69

3.3.11. Multivariate regression model to establish significant explanatory variables contributing to direct costs in NAFLD

Direct costs in NAFLD can be influenced by the patients' characteristics. While some characteristics are directly related to the target condition, for example, disease stage, other aspects such as age or metabolic profile, can be related to costs regardless of liver condition, as 'confounders'. A multivariable regression model was generated in which the dependent variable was total cost per patient. As independent (explanatory) variables, the models included disease stage, plus those variables that were considered potential confounders. From the regression model ***(Table 3.17)*** a statistically significant increase in the mean total direct costs in patients was observed in patients with each unit change in fibrosis (0.322, p=0.001). This model examines the true effect of fibrosis stage on cost holding all other confounding variables constant. A similar trend is observed with the number of OPD appointments (p=0.042). An inverse trend can be seen with dyslipidaemia (p=0.026), indicating that its presence as a co-morbidity is associated with lesser direct costs.

Table 3.17: Multivariate regression model in NAFLD costing

	Direct costs		
Explanatory variable	**Co-efficient**	**Z**	**p-value**
Age	0.083	0.920	0.359
BMI	0.046	0.534	0.594
Suspected NAFLD	-0.167	-1.827	0.070
Time to first OPD	-0.137	-1.616	0.109
Number of OPD	0.177	2.053	0.042
Patient metabolic profile			
Type II Diabetes Mellitus	0.107	1.091	0.278
Hypertension	0.032	0.307	0.759
Dyslipidaemia	-0.248	-2.262	0.026
Histological Stage			
Fibrosis Stage	0.322	3.569	0.001

3.4. DISCUSSION

Useful data generated in this study includes descriptive data on the spectrum of hepatology OPD reviews conducted over a 4-week period, where patients were profiled in terms of disease aetiology and for new referrals, on the basis of 'reason for referral'. This information was gathered in parallel with the range of medical investigations requested at this out-patient encounter. We chose to first characterise a typical hepatology OPD to determine the burden of disease afforded by NAFLD in an OPD setting as compared to other aetiologies of liver disease. In order to assess 25% of the population affected by NAFLD, the initial study was then complemented by a follow-up study, where a bottom-up micro-costing analysis provided estimates for the average OPD costs for a NAFLD, ALD, NAFLD cirrhotic, ALD cirrhotic, known or suspected NAFLD patient.

This study confirms that both NAFLD and ALD have a high prevalence at referral in the UK. This observation aligns with OPD trends previously described in the US. The take home message from this study is that the direct medical costs associated with NAFLD (with an estimated global prevalence of 25%) are substantial and increase exponentially with the presence of advanced disease.

3.4.1. Rationale for study

Information on patterns of utilization of services in hepatology outpatient departments is fundamental to inform policy makers about resource priorities and potential obstacles in the delivery of this service. Little published information on OPD resources utilisation and the incumbent economic burden of NAFLD is available despite the increase in peer-reviewed NAFLD publications (365).

The reported case-mix of hepatology consultations in this study is in keeping with current trends; NAFLD (24%), ALD (14%) and chronic viral hepatitis (9%). This case-mix reflects the predictions made in a recent Lancet report stating that the combination of alcohol, viral and obesity related liver diseases are predicted to result in 63,000 preventable deaths over the next 5 years (113).

Current medical practice tends to dichotomise a diagnosis of fatty liver disease into one of two common forms, Alcoholic Liver Disease (ALD) and Non-Alcoholic Fatty Liver Disease

(NAFLD), based on a widely-adopted threshold for alcohol consumption set at 20g/day for women and 30g/day for men (42, 366, 367). Both conditions are associated with maladaptive lifestyle factors (42, 52, 368, 369). These two population subsets were useful to compare in this study in terms of cost as the combined environmental challenges of alcohol consumption and calorific excess/metabolic risk oftentimes overlap and interact to modify progression of liver disease. Overlap was identified in approximately 9% of patients (20/217) in this study group.

The burden of ALD is hard to accurately quantify. Current US prevalence is estimated to be steady at 2-2.5% (370, 371). However, in Europe, while some countries have reported a decline in the burden of ALD, others have reported a recent increase in mortality from ALD cirrhosis (332). The scale of the NAFLD epidemic is by far the most alarming, and the rapidly expanding formulary of anti-fibrotic drugs undergoing Phase III clinical trials and impending regulatory assessment makes it an area of chronic liver disease of great interest in many health care systems. The 25% estimate for the global prevalence of NAFLD is widely accepted (8, 17), however regional differences were described by Younossi et al with the highest disease rates being reported in South America, the Middle East, Asia, the USA and Europe (372). To date, real world cost data for NAFLD remains sparse internationally and was poorly documented in the UK (111).

3.4.2. Comments on the frequency of advanced disease observed in this study

Most of the morbidity and mortality associated with liver disease occurs in those with advanced fibrosis (373). In this study, 86% of the ALD cohort and 40% of the NAFLD cohort had cirrhosis. This is not an uncommon demographic in a tertiary facility, as Chronic Liver Disease (CLD) care guidelines advocate referral for HCC surveillance when there is a clinical suspicious of advanced fibrosis/cirrhosis (374).

In this study, the multivariate analysis preformed showed that advancing disease was predictive of increased costs in NAFLD. This is in keeping with US studies that report a 90% and 70% increase in total charge and payment costs in NAFLD with the development of cirrhosis (375). A recent NAFLD meta-analysis reported that 9% of NASH patients will progress to cirrhosis each year (8). However, unless obesity interventions are laboured this trend looks likely to increase with a resultant increase in the burden of advanced disease. This trend is further perpetuated by the increasing prevalence of obesity observed in the pediatric

population (0.7% in 1975 to 5.6% in 2016 in girls, and from 0.9% to 7.8% in boys) (376). Furthermore, animal models of juvenile fatty liver disease have also shown that the progression from hepatic steatosis to NASH is more rapid and aggressive in children than in adults and increases the risk of cirrhosis and HCC in later life (377).

In contrast, only a small fraction of the ALD population with continuing levels of alcohol excess progress to cirrhosis (378, 379). In terms of progression to advanced disease, as reflected in our data, ALD progresses more rapidly than NAFLD. Studies have shown that over a median follow-up time of 178.27 months, 20% of ALD patients progressed to cirrhosis compared to only 7% of NAFLD patients (379).

3.4.3. Comments on the frequency of mild disease observed in this study

Unlike ALD, where 86% of the patients presented with established cirrhosis, the non-cirrhotic case load in NAFLD is reported at 60%. In NAFLD, current practice is evolving to promote the identification and referral of patients with NAFLD at an earlier stage in the natural history of the disease through community awareness programmes e.g. British Liver Trust, "Liver disease- a clinical priority for Primary Care" (380). It is reassuring to observe that this advice appears to be practiced in primary care. In the suspected NAFLD cohort, of the 49 referrals, only 11 were confirmed cirrhotics and of the 31 'undetermined' subtypes, >50% had fibroscan readings reassuring for the absence of advanced disease. Interventions aimed at NAFLD and ALD with mild disease are critical for health care cost containment going forward as to be cost-effective there is a need to identify patients prior to their presentation with the complications of end-stage liver disease which generates significant costs relating to HCC screening and hospitalisations. In an OPD setting, there exists an opportunity when discharging or following up 'mild disease' patients to provide information on steps to slow disease progression. It may be useful to address this in NAFLD information sheets as several recent studies in the US have shown that clinicians in both general and specialized practice have limited knowledge regarding NASH and health authorities in the US are currently supporting the need for education in this area (104).

3.4.4. Cost drivers in NAFLD/ALD

This study demonstrates that the direct medical cost associated with both NAFLD and ALD care in the UK are substantial. This is consistent with current literature. A recent US study

reported a 5% annual cost growth rate in NAFLD out-patient expenses (111). As our data collection period was over a 12-month period, we were not in a position to offer annual cost growth rate trends in the UK.

As observed in our data, the direct costs associated with NAFLD have been reported to increase exponentially with progression to advanced disease (111, 381, 382). In addition to the cost burden associated with advanced disease, one can predict that considering the total number of patients with NAFLD in the UK, these costs have the potential to become enormous (Approximately 25% of the UK population have NAFLD, with an expectation that 10-12% of these will progress to advanced disease/cirrhosis).

Total ALD and NAFLD cohorts; the principal cost driver in both groups was the frequency of hospitalisation episodes. A further cost contribution was associated with imaging and serological investigations. When hospital costs were excluded, total costs were greater in the NAFLD than ALD cohort most likely reflective of the increasing number of referrals in the NAFLD group. The mean annual cost per patient was almost 2.5 greater in the ALD group compared to the NAFLD group (£565 versus £1361).

NAFLD and ALD cirrhotics; The principal cost driver was again the frequency of hospitalisation episodes. This resource use was significantly higher in the ALD group versus NAFLD group. Within the total NAFLD group, cirrhotic patients were responsible for 88% of hospitalisation days and 76% of imaging investigations. This in in keeping with trends observed in US data which also reports an increase in the number of NAFLD related hospitalisations with costs increasing from $249,884 to $326,403 over a time period of 8 years (383). In this study, the mean annual cost per ALD cirrhotic patient was almost double the mean annual cost of a NAFLD cirrhotic patient (£927 versus £1592). Unfortunately, despite the prevalence of ALD being reported as relatively stable, the incidence of ALD is increasing, particularly in certain subgroups such as females and it is likely that this is a trend which may escalate in the future. Similar to US data, in this study ALD costs are substantial with mean costs associated with ALD greater than those observed for NAFLD.

Known and suspect NAFLD groups, the known NAFLD group, which included the majority of the cirrhotic subset, had total projected annual costs almost triple that of the suspected NAFLD cohort (£691, 084.92 versus £244,908.36). This prediction was in line with the multivariate regression model which observed advanced disease as a significant factor in predicting direct medical costs (p=0.001). The mean annual cost for a patient with known

NAFLD was almost double the mean annual cost of a patient with suspected NAFLD (£416 versus £647). This is in line with a study conducted by Baumeister et al in data derived from the German SHIP study. This group observed that patients with a "suspected" diagnosis of NAFLD (hepatic steatosis on imaging, deranged LFTS) increased overall costs by 32% with a 15% increase in outpatient costs and a 38% increase in inpatient costs (381).

This study provides a useful breakdown of costs reflective of suspected and established NAFLD diagnoses. These figures represent useful disease costing building blocks as it is highly likely that an increase in OPD costs due to NAFLD is on the horizon given that 82% of the suspect NAFLD cases in this study were over the next 11 months confirmed as NAFLD. Currently, the annual cost associated with all incident and prevalent cases of NAFLD in the Europe is estimated at 35 billion euro. However, if estimates are adjusted to parallel the expected trajectory of obesity in the future the 10 year burden of NAFLD could be estimated to be close to 334 billion euro (8).

A point to consider when studying costing data, and a potential factor to elucidate as the number of COI studies in NAFLD escalate, are do the increasing number of referrals to tertiary centres actually correlate with a decrease in patient mortality. Interestingly, in the US, despite an increase in OPD interactions, the mortality rate has remained stable at 2.84% (384).

Comparison to US data Younossi et al. (using an incidence based model) generated figures for the lifetime cost of NAFLD ($222.6 billion), and the cost of the advanced NASH population ($95.4 billion). Similar to our study this group did not include costs of comorbidities, nonmedical costs, or the societal costs of NASH (385) In a follow-up study, an interesting cost break-down was provided by the same US group. They delineated total liver related costs of NASH with T2DM versus NAFL with T2DM ($95 per-person-year versus $2,275 per person-year). In both subgroups, the majority of costs are related to the diabetes care. However, the costs for non-progressive NAFLD were not insignificant- liver-related health care for this group still accounts for $13.7 billion per year (annual liver check-ups and evaluations). These figures did not account for complications of advanced liver disease which was identified as a main cost driver in our study (386). Similar to the findings in this study, this US data indicates the large economic burden of NASH and advanced NASH and the urgency to plan for future care delivery.

3.4.5. Metabolic co-morbidities

In NAFLD patients, the presence of metabolic risk factors accelerates disease progression (387). Documentation and optimisation of metabolic risk factors is critical in NAFLD where cardiovascular disease is the most common cause of death (378). Profiling of patients in the initial consultation provided valuable information when controlling for confounders on the multivariate regression model powered to predict the main factors influencing direct costs in NAFLD.

As predicted, in the NAFLD cohort, observed cases of T2DM, hypertension and dyslipidaemia were higher (48%, 55% and 56%) than in the ALD cohort (11%, 15% and 11%). Although metabolic co-morbidity management was not costed in this study, previous studies have reported increasing costs with the presence of metabolic co-morbidities (obesity and hypertension), although this was not observed in the multivariate regression model generated (388).

Obesity: In the NAFLD subgroup, the average recorded BMI was 34 kg/$m^{2.}$ Whilst obesity *per se* is not directly associated with more advanced hepatic fibrosis stage in patients with NAFLD (389), a higher BMI has been found to associated with more advanced hepatic fibrosis in ALD (339, 340). Several epidemiological studies support the view that there is a strong causal relationship between consumption of a diet high in fat (and/or presence of T2DM), the consumption of alcohol and progressive liver disease (335, 336, 338, 390, 391). Amongst patients with high alcohol consumption, obesity is an independent risk factor for acute alcoholic hepatitis and cirrhosis (339, 340). It has been demonstrated that alcohol and obesity/T2DM confer quantitative and qualitative changes to the intestinal microbiome and impair the intestinal barrier thus promoting steatohepatitis and fibrosis (392, 393). Obesity and alcohol also reduces adipokine production in visceral adipose tissue. Adiponectin (anti-fibrotic) is reduced in individuals with obesity or sustained high alcohol consumption (394-396). One can speculate that documented high levels of obesity will likely lead to faster progression to advanced disease and greater future costs.

T2DM: A large cohort study found that IR/T2DM was an independent predictor of overall and liver-related mortality in ALD and NAFLD patients (10, 24, 397-400). In this study, the level of T2DM in NAFLD was 48%, marginally lower than that observed in the US among cirrhotic NAFLD patients (60%) (372). As expected, lower rates of T2DM were reported in the ALD group (11%). In support of this observation, there is epidemiological evidence to suggest that

consumers of moderate levels of alcohol have a lower risk of T2DM compared to non-drinkers (401-403). Findings from a meta-analysis based on 13 prospective studies, suggested wine consumption was associated with a significantly lower risk of T2DM: 20% risk reduction at 20–30 g pure alcohol/day (404). However, a critical analysis report interrogating data from case control and cohort studies concluded that the protective effects of moderate alcohol intake often lack a sufficiently clear definition of the dose of alcohol consumed or an accurate estimate of the size of the derived protective effect, with publication bias responsible for a potential overestimation of the reported effects (405).

3.4.6. Ancillary resource utilisation

3.4.6.1. Serological investigations

Serological investigations have a high uptake in all cohorts and account for a large fraction of expenses. Their main use is in assessing liver synthetic function, particulary in advanced disease. They remain of limited value in detecting alcohol excess or diagnosing NAFLD (406-410). AFP costs are also high in both groups. This serological investigation is of partciular use in NAFLD, where HCC can develop in the absence of cirrhosis (411). Lipid and glucose measurements generated considerable costs in the NAFLD subgroup which is not surprising given that the literature reports elevated serum lipid profiles and glucose concentrations in 25–75% of NAFLD patients (412). Monitoring of metabolic risk factors is appropriate in NAFLD where the presence of metabolic risk factors has been reported as more common among people who develop HCC (37.1%) compared to 17.1% in patients without HCC (413).

3.4.6.2. Imaging

Patients with NAFLD and ALD are at increased risk of developing HCC and justification for surveillance by means of 6 monthly USS has been justified as per data obtained from the SEER (Surveillance, Epidemiology, and End Results database) reporting a 4-fold increase in the HCC risk in patient with ALD and 2.5-fold increase in patients with diabetes/obesity (414). Direct costs pertaining to imaging are significant in each of the cohorts. It has been cited that of the 9% of NASH patients who progress to cirrhosis each year, 2.6% will likely develop HCC (8). As anticipated, an increased number of surveillance USS and more complex imaging investigations were recorded in the ALD group. Intensive surveillance where the index of clinical suspicion is high is supported by the literature, where in a case-

controlled study by Lok et al, the authors reported an approximate 6-fold increased risk of HCC with alcohol consumption (compared to a 5-fold risk with tobacco and 4-fold risk for obesity) among Americans with cirrhosis (415).

3.4.6.3. The importance of staging investigations

Adverse outcomes in NAFLD are related to histological subtype, patients with advanced fibrosis and NASH in addition to steatosis are at an increased risk of liver mortality (82). Histological evaluation is considered the gold standard to stage NAFLD patients (116). However, given the high prevalence of NAFLD, widespread use of liver biopsy as a diagnostic tool is impractical. Most expert guidelines recommend liver biopsy for NAFLD patients at high risk for NASH and/or advanced fibrosis, if there are discordant non-invasive tests or if NAFLD is suspected as a co-existing chronic liver disease (121-123). In this study, liver biopsy was performed in only 9/138 patients seen in hepatology OPD over a 4-week period (projected estimates ~108 liver biopsies for NAFLD per year). Lower numbers are reflective of the increased use of ultrasound based elastography techniques (e.g. Fibroscan^{TM}, ARFI, SuperSonic^{TM}) shown to be safe, quick, and cheap methods to radiographically assess for advanced fibrosis (171). In the NAFLD cohort, 63 patients were "undetermined". However, utilising non-invasive techniques (Fibroscan^{TM}) in 53% of these patients, they were deemed low risk for advanced fibrosis and did not proceed to biopsy. However, the Poisson regression provided evidence that the performance of liver biopsy was predictive of a lesser number of follow-up hepatology OPD appointments (IRR 0.617, p=0.001). This highlights the importance of comprehensive staging investigations to efficiently and appropriately manage NAFLD patients.

In contrast, liver biopsy is seldom performed in ALD. Despite this current trend, it is worth noting that only biopsy data can provide information on the different subtypes of ALD. A French study of 1604 biopsies reported that 11% of ALD patients had cirrhosis with alcoholic hepatitis, 15% had cirrhosis without, 12% had normal histology, 18% had fibrosis, while 25% had simple steatosis (339). The presence of hepatitis affects prognosis in ALD. This spectrum of ALD disease stage is in contrast to our cohort where 86% were cirrhotic, a large fraction of which was diagnosed by radiology thus, the presence of alcoholic hepatitis was not documented. Patients with alcoholic hepatitis have a worse prognosis, especially in the

setting of cirrhosis with only 35% of this population still alive at 48 months after an acute presentation (416).

3.4.7. Dietician referrals

The relatively low number of referrals made to dietician services for NAFLD (228 de novo referrals in 1 year) and ALD (0) is not ideal. Epidemiological and experimental studies indicate that both the quantity and the type of dietary fat can influence ALD (396, 417) and NAFLD pathogenesis (418, 419). In NASH, diet coupled with exercise can produce significant weight loss in addition to improvements in histologic components of NASH (131, 420, 421). Although an upper limit for the amount of weight loss has yet to be established, current best evidence suggests that weight loss of at least 7 % is necessary to improve hepatic inflammatory activity and weight loss (10 %) is necessary to improve fibrosis in NASH (422). In the NAFLD cohort, 12% of patients had a BMI <25 (suggesting that they had lean NAFLD versus may have adhered to prescribed lifestyle interventions pertaining to weight loss). 62% of the ALD cohort were now abstinent from alcohol. Of concern is the fact that it has been shown that patients often develop obesity after drinking cessation. A variant in *FGF21* was found to be associated with increased consumption of sugary snacks and increased alcohol intake, suggesting that the FGF21 hormone secreted by the liver may influence nutrient choices (423). Chemically, there are similarities between alcohol abuse and obesity centred around dopamine activity (424). A reduced numbers of dopamine (D2) receptors in the brain of obese individuals and those with chronic alcohol abuse have been observed (425, 426). This receptor deficiency is thought to drive a compulsive engagement in pleasurable behaviours, such as alcohol use and eating i.e. a “Reward Deficiency Syndrome” (42, 427, 428) providing an argument for behavioural restructuring techniques in both populations. However, despite the level of evidence that would suggest the potential value of nutritional guidance in NAFLD and ALD care pathways, this is something that is not routinely offered, largely due to financial constraints.

3.4.8. Current clinical burden, future implications

Chronic liver disease has traditionally been a tertiary speciality, however the chronic nature which is observed in NAFLD necessitates a shift to patient follow-up in a primary care

environment. This is possible as most patients with NAFLD steatosis do not appear to progress therefore per se, do not necessarily need referral to a specialist liver centre (429). However, at baseline both ALD and NAFLD are characterized by substantial interpatient variation in disease severity and risk of progression to cirrhosis (53, 369). This poses difficulties in the community where fibrosis screening services are limited and may be a reason we see a large number of referrals with suspected NAFLD as primary care physicians are anxious for a comprehensive fibrosis stage assessment.

NAFLD and ALD already constitute a heavy primary care burden because of the co-existence of co-morbidities including psychiatric disease, metabolic syndrome and substance abuse issues (373). Within NAFLD, the new to review case ratio was 1:2. Over the next 12 months, patients waited on average 210 days for their next appointment and had an average of 2 OPD appointments per year. This patient burden and schedule of follow-up alerts policy makers of the need to increase capacity of existing services. Strategies are being promoted in a recent blueprint by the Lancet whereby they are trying to establish acute liver services in district general hospitals and link them with regional specialist centres for more complex investigations and treatment and are actively canvassing for increased provision of medical and nursing training in hepatology(108).

Integrating NAFLD/ALD monitoring with primary care may be one way to reduce the demands on tertiary hepatology services while simultaneously increasing patient satisfaction, convenience and clinic attendance (430). Currently, nursing services in NAFLD and ALD clinics serve to record patient vital measurements; approximating 600 hours for NAFLD and 252 hours for ALD over a 1 year period. Expanding their role in NAFLD/ALD assessment may be necessary as a strategy to meet increase service provision demands (431) . With the imminent arrival of anti-fibrotic drugs on the market, it is likely there will be an increased referral of patients with fatty liver as previously no treatment outside lifestyle intervention was available which will add to the already considerable OPD burden.

3.4.9. Strengths and Limitations

Study population This study is unique in that it provides a detailed cost analysis of two lifestyle related diseases. A recent literature review has confirmed that this study obtains more complete cost estimates than any of the previous studies to date. Both cohorts involved in this study benefited from precise histological staging as per liver biopsy reports,

particularly in the NAFLD cohort as a large number of NAFLD patients had undergone liver biopsy previously. However, this may not be reflective of future practice with the widespread availability of transient elastography to pre-screen for liver biopsy suitability.

Study Design This study was carried out at a single tertiary facility in the UK, which is a potential limiting factor regarding the external validity of the results obtained which may be subject to referral bias as only more severe patients are referred for management. This study is a retrospective prevalence based study, therefore it is unable to be used to follow patients from initial diagnosis. As the study design was retrospective, it is possible some patients with moderate disease and compensated cirrhosis developed more advanced disease over the course of 12 months. However, considering the slow progression of NAFLD the numbers, if any are likely to be small. This study provides a framework that can be adapted in other healthcare systems performing similar COI studies. The large, heterogeneous snapshot of NAFLD and ALD patients is reflective of the case-mix observed in hepatology practices in the UK and provides useful information to guide health care policy makers to decide the most sensible and strategic investments. The strategy adopted was micro-costing, which is the most methodically accurate way to assess unit costs but is seldom done in clinical practice owning to time constraints. There are also some limitations regarding costing, which was based on the assumption that the volume of patients reviewed for the month of October would be reflective of practice each month.

Potential collaboration This study constitutes the UK arm of the European CONSTANS study. This study involves 4 additional participating countries (France, Italy, Switzerland and Germany) to study the prevalence of NAFLD in specialized liver clinics versus other reasons for medical consultation. This study is powered from a European perspective and its completion will reflect a work involving international expertise from clinical, economic and scientific domains to improve NAFLD care.

Useful to aid the introduction of new drug therapies the data provided by COI studies is becoming more pertinent in light of new anti-fibrotic drugs on the horizon in NAFLD where it may supplement pharmaco-economic analyses to assess the cost-effectiveness of these novel interventions.

Deficiencies in data collection This study involved sub-optimal recording of alcohol consumption which is an issue in all hospital based NAFLD and ALD studies (355). The precise recording of both the quantity and type of alcohol is imperative and was not

accurately documented in this study. The number of days per week on which alcohol is consumed, the typical number of drinks per day, and evaluation for the presence of episodic heavy drinking should be performed to characterize the moderate alcohol user. For example, in a recent Danish study, the amount of alcohol consumed per session has been proposed to be more important in relation to risk of developing T2DM than drinking frequency (432).

3.4.10. Future directions

The literature would suggest that both ALD and NAFLD may co-exist to varying degrees in a significant number of patients. In light of the rising prevalence of metabolic syndrome, NAFLD and ALD, the plausible concept of "dual-aetiology fatty liver disease" may serve as a more tractable diagnostic category that reflects the co-existence of these diverse processes in patients with fatty liver disease and higher-than-recommended alcohol consumption that however falls short of a clear ALD diagnosis. The costing data generated in this study may serve as a platform to estimate costs in this disease subtype, if it receives recognition as a disease state in the future.

Evidence for a shared pathophysiology between ALD and NAFLD may be derived from genetic studies, which have demonstrated that the severity and progression of both NAFLD and ALD are influenced by a number of the same genetic variants (201-205, 433-437). Genetic screening may be an option going forward and it may be interesting to use this data as a platform to assess the cost-effectiveness of this screening tool.

Future studies should have clear guidance with relation to the documentation of alcohol consumption. As evidenced in this study, the data on alcohol consumption is quite poor. There is now considerable evidence that screening tools are more effective than biochemical markers of alcohol use, in particular the Alcohol Use Disorders Identification Test (AUDIT), developed by the World Health Organisation (438, 439). These screening tools can be used effectively to identify and intervene with risky alcohol consumption in hepatology clinics (440). However, they have variable sensitivity depending on the population being screened and the validity of alcohol screening tools amongst patients with

combined alcohol and metabolic risk factors is a field which requires further research before it can be recommended as best practice.

3.5. CONCLUSION

ALD and NAFLD are two of the most frequently occurring liver diseases worldwide. NAFLD impacts all age groups, its prevalence increasing with age (429). The current strain on service provision is escalating based on worrying trends observed in the paediatric population (376, 377). Unfortunately, the possibility of a cure for both ALD and NAFLD, both lifestyle associated liver diseases is unlikely. There will inevitably be challenges in many health care systems to support this expansion over the coming years.

In particular, NAFLD is undergoing a revolution in terms of therapeutic options. This is a special time where it is possible to conduct COI studies in NAFLD before the introduction of anti-fibrotic agents which may have a budget impact in the UK. The outcomes of this study emphasise the need to identify public health factors that policy care makers can address to develop population or community based interventions to contain NAFLD and ALD growth.

Personalised treatment strategies will also be fundamental to improve clinical outcomes in the most cost-effective manner. To date, voluntary restraints by the food and drinks industry have had little effect on disease burden, and concerted regulatory and fiscal action by the UK Government may have an important role to play. In a study predicting obesity levels in the US in 2030, it is predicted that even if through simple health promotion efforts, current obesity levels could be maintained at current levels, there would be potential to save the US health economy 440 billion over the next two decades (441). This potential saving could then be re-invested in such avenues as anti-fibrotic therapy development which have the potential to improve patient morbidity and mortality.

CHAPTER 4.

AN INITIAL EXPLORATION OF PROTEOMIC BIOMARKERS IN NASH

4.1. INTRODUCTION

4.1.1. NAFLD Pathogenesis

Non-alcoholic fatty liver disease (NAFLD) represents a continuum of liver injury ranging from simple steatosis affecting ≥5% of hepatocytes (NAFL) to non-alcoholic steatohepatitis (NASH), characterised by the presence of steatosis, lobular or portal inflammation, hepatocyte ballooning and fibrosis (52). Fibrosis is cited as the key histological feature predictive of clinical outcomes (53, 54, 56, 128). In simple terms, liver fibrosis represents a chronic repair process in response to persistent liver injury (442). ECM remodelling is a key process in tissue homeostasis however under pathological conditions, imbalanced ECM remodelling results in fibrosis and consequently the release of tissue and pathology specific turnover products into the systemic environment (443, 444).

4.1.2. Non-invasive assessment of liver histology

Routinely measured transaminases correlate poorly with liver histology. Their diagnostic accuracy however is enhanced by their inclusion in biomarker panels, for example the validated NFS, APRI, and FIB4 indices (164, 165, 445). However, these scores were derived and validated in cohorts recruited from tertiary liver centres with a high prevalence of advanced disease therefore when applied to the general population with less severe disease their performance declines (165). Other caveats, such as the effects of age have been corrected for with the development of age-specific cut-off for the NFS and FIB4 (446). Genetic factors have little predictive value despite having important roles as disease modifiers (205). Transient elastography (Fibroscan ^{TM}) strongly correlates with advanced fibrosis stage, hepatic venous pressure gradient (HVPG) and survival however their performance is inadequate in common clinical scenarios such as mild fibrosis, ascites, cholestasis, obesity and volume overload. Its mode of application is also one dimensional therefore similar to liver biopsy, it is also liable to sampling error (447). The future gold standard for fibrosis assessment is likely to be magnetic resonance elastography (MRE). This modality obviates sampling error by assessing the entire liver, however its universal adoption has been hampered by its high cost(448). Despite the attraction, the non-invasive imaging techniques described do not reflect disease activity and prognosis in contrast to dynamic biomarkers (449). A clear mandate for the development of biomarkers reflective of 'real time' fat accumulation, necro-inflammation and fibrogenesis in liver disease exists.

4.1.3. Limitations of liver biopsy

Liver biopsy is the reference standard for fibrosis assessment. However, the increasing prevalence of NAFLD necessitates a shift from histology towards the development of non-invasive assessments. Liver biopsy remains the established but imperfect 'gold standard' investigation being invasive, resource intensive, prone to sampling error and carrying a small but significant risk of complications (114). It is discussed in detail in chapter 1.

4.1.4. PIIINP the first well characterised direct serum biomarker

Type III collagen is one of the main components of the interstitial matrix involved in inflammation-fibrosis tissue injury. PIIINP, an amino terminal peptide of type III procollagen has been extensively studied as a marker of liver fibrosis. Undisputed is the relatively linear relationship between PIIINP and the grade of NASH. In a study by Tanwar et al, PIIINP levels discriminated the majority of patients with simple steatosis from those with steatohepatitis (AUROC 0.82-0.84). Secondly, within a cohort of patients with NAFLD comprised of all stages of fibrosis, PIIINP levels allowed discriminating the majority of patients with NASH or advanced fibrosis from those with steatosis (AUROC 0.85-0.87) (155). PIIINP has been incorporated into the NICE endorsed Elevated Liver Fibrosis score (***ELF^{TM} score)*** (175). The ELF score has demonstrated variable performance in identifying NASH patients with advanced fibrosis; speculation in the literature would suggest the following causative factors. (1) PIIINP is non-specific for protein formation or degradation. PIIINP assays assess the pro-peptide of type III collagen which is cleaved off pro-collagen during collagen maturation. However, this process may be incomplete generating a thin collagen fibril with abnormal cross-links susceptible to rapid metabolic turnover (450, 451). (2) A number of assays for PIINP measurements have been developed often incorporating antibodies not specific to the epitope at the protease cleavage site (452). In a paper employing the ADVIA Centaur platform to assess PIIINP, a 2 fold difference in the measured levels were reported when a radioimmunoassay (UNiQ) was used compared with the new ADVIA Centaur immunoassay which has been tested as a component in the ELF test by Siemens health care diagnostic (452). The lack of a standardised PIIINP assay and the fact that the PIINP antibodies used are often not disclosed (therefore may not be specific to the pro-peptide cleavage site) represents problems with consistency of performance. To improve biomarker performance, neo-epitope based biochemical markers measured in serum or plasma have

been characterised where neo-epitopes represent a unique fingerprint of proteolytic cleavage of the protein and may be used to identify pathologically affected tissue (444). Noteworthy examples include fragments derived from type I collagen as markers of bone resorption (CTX1), ICTP of prognostic value in myeloma, and C1M in rat liver fibrosis (453-455). A neo-epitope specific competitive Enzyme-linked immunosorbent assay (ELISA) towards the N-terminal pro-peptide of type III collagen (PRO-C3) to assess true formation has been developed and will be assessed as one of the biomarkers in this study.

4.1.5. Biomarkers that reflect fibrogenesis and fibrolysis

Progression to cirrhosis is associated with a 10 fold increase in fibrillar collagens (type I, III and V) and up to a 6 fold increase in type IV collagen resulting in increased liver stiffness (190). A non-specific molecular signature of this transition could be summarised as an increase in type I over type III collagen with a decrease in type VI collagens (195). Fibrillar collagens are synthesised with pro-peptides molecules which undergo proteolytic cleavage before collagens can form triple helices in the ECM. These pro-peptides molecules and their subdomains can be quantified as serum biomarkers for ECM formation, turnover or degradation (193, 197) As discussed above, 'Protein fingerprint' technology has been exploited in the development of novel biomarkers focusing on the identification of such pathologically modified proteins. A number of assays have been developed to quantify collagen fragments reflecting collagen formation (PROC3, PROC6 and PROC4) and degradation (C3M and C4M) in preclinical (animal) models and patients (51-53).

4.1.6. Formation Biomarkers- PROC3- PROC4 and PROC6

PROC3 is a pro-peptide of type III procollagen released by the protease ADAMTS-2 during collagen maturation (456). Using monoclonal antibodies incorporated into an ELISA to target the N-protease cleavage site it is possible to assess collagen formation only (456).
PROC4 represents the 7S domain of type IV collagen. Excessive accumulation of type IV collagen with abnormal deposition of basement membrane material around the sub-endothelial sinusoids and in the Space of Disse is a hallmark of advanced fibrosis (457, 458). Serum PROC4 measurements are representative of systemic collagen type IV formation (458, 459).

PROC6 is classified biologically as an adipokine called endotrophin which is highly expressed in adipose tissue (460). Measurement of PROC6, precisely the COOH-terminal pro-peptide of the alpha-3 chain of type VI collagen may be valuable in evaluating NASH as it is representative of the NASH hallmarks of fibrotic remodelling and metabolic derangement.

4.1.6.1. Degradation Biomarkers C3M and C4M

C4M; Fibrogenesis involves the disruption of cell basement membranes (BMs) which are rich in type IV collagen, consisting of six distinct alpha chains, α1-6(IV). Sand et al have developed a competitive C4M12 α1 (C4M) ELISA to target a neo-epitope associated with the α1(IV) chain as a marker of BM degradation during liver fibrosis (199).

C3M; In parallel to BM destruction, Type I and III collagen levels undergo an 8 fold increase (461), with a surge in gelatinase MMP-9 reflective of increased proteolytic activity (462, 463). Researchers, Barascuk et al have developed an ELISA for an MMP-9 proteolytically revealed neo-epitope of type III collagen, C3M as a marker of collagen degradation and a potential fibrosis biomarker (464). However, to date, C3M and C4M have failed to identify fibrosis regressors (465).

4.1.6.2. ECM Related Biomarker -LOXL2

In fibrogenesis, collagen cross-linking is mediated by LOXL2, which functions as a copper-dependent matrix metalloenzyme (466). In liver fibrosis samples, elevated levels of lysyl oxidase-like-2 (LOXL2) expression has been observed, with its upregulation limited to fibrotic areas (466, 467). An ELISA targeting this enzyme may be a surrogate marker of collagen and therefore valuable as a fibrosis biomarker. When considering this as a potential biomarker, it is worth noting that a monoclonal antibody against lysyl oxidase-like 2 (Simtuzumab) failed to decrease liver collagens in two phase 2b clinical trials in NASH patients with advanced fibrosis/cirrhosis (468).

4.1.6.3. Wound Healing Biomarkers- FPA and VWF

A strong correlation has been established between collagens, altered haemostasis and fibrosis (469).

Fibrinopeptide A (FPA) is a by- product of thrombin-induced proteolytic cleavage of fibrinogen. In a recent publication by Xin et al, the group established a peptidomic pattern

that could distinguish NAFLD patients from their twin controls (470) and proposed FPA as a potential biomarker of NAFLD severity (471).

Von Willebrand factor (vWF) is a protein involved in platelet adhesion and aggregation (472, 473). In fibrogenesis, secondary re-epithelisation of damaged vascular endothelial cells occurs when Von Willebrand factor binds to the exposed fibronectin rich ECM (474). The consequent clotting process involves platelet aggregation and micro-thrombotic events culminating in an increase in portal pressure and fibrosis propagation. The literature to date has established vWF-Ag as a valuable marker for prediction of varices, portal hypertension and mortality in patients with liver cirrhosis (473, 475).

4.1.7. Study Objectives

The objective of this study is to validate the use of proteomic biomarkers to predict histological liver disease severity.

- A preliminary assessment of a panel of collagen neo-epitope biomarkers will be conducted to determine their relationship to (1) fibrosis stage and (2) NASH in a NAFLD cohort representative of the full disease spectrum. This study aims to identify the most competitive collagen fragment to undergo further biomarker utility studies.
- An additional sub-study will be performed to investigate LOXL2, FPA and VWF as potential biomarkers in NAFLD.
- All biomarker performances will be compared to validated fibrosis indices.

4.2. MATERIALS AND METHODS

4.2.1. Study Design and Participants

Figure 4.1 shows the flow of patients through the study. Patients were recruited as described in chapter 3.

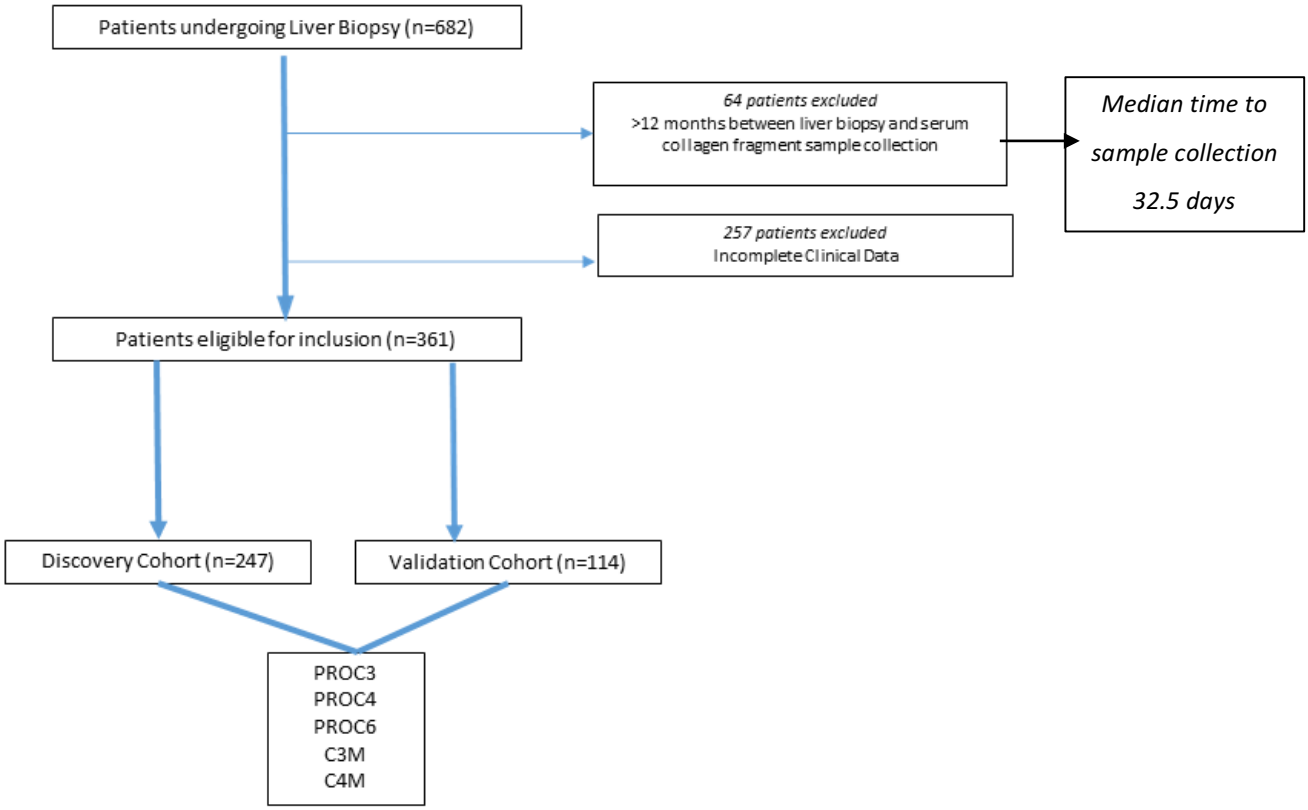


Figure 4.1. Study Design

4.2.2. Clinical and laboratory assessments

Clinical and laboratory assessments were performed as described in chapter 3. PROC3 PROC4, PROC6, C3M, C4M, LOXL2, FPA and VWF were assessed using competitive ELISAs (Nordic Bioscience A/S, Denmark) measured by experienced technicians unaware of any associated clinical data (456, 476).

4.2.3. Calculation of Fibrosis Indices

Fibrosis indices were calculated as described in their validation publications (174, 477-480).The AST to ALT ratio was calculated as AST/ALT. The APRI was calculated as AST (IU/l)/ (upper limit of normal)/platelet count ($\times 10^9$/litre) $\times 100$. The BARD score was composed of 3

variables: AST/ALT ratio >0.8-2 points; a BMI ≥28 – 1 point; and the presence of diabetes – 1 point. The possible score ranges from 0 to 4 points. The FIB-4 score was calculated: age × AST (IU/l)/platelet count ($\times 10^9$/litre) $\times\sqrt{}$ALT (IU/l). The NAFLD fibrosis score was calculated according to the following formula: –1.675+0.037×age (years)+0.094×BMI (kg/m^2) +1.13×impaired fasting glycaemia or diabetes (yes=1, no=0) +0.99×AST/ALT ratio –0.013×platelet ($\times 10^9$/litre) –0.66×albumin (g/dl). The ADAPT score was calculated:

$$\text{ADAPT} = \exp\left(\log 10\left(\frac{\text{Age}\times\text{PROC3}}{\sqrt{\text{Platelets}}} + \text{Diabetes}\right)\right).$$

4.2.4. Histological Assessment

Histological assessment was performed as described in chapter 3. To reduce the element of inter-observer variability, over half of all biopsies (237, 66%) in our study were centrally read by expert member of the EPoS Histopathology Group (DT).

4.2.5. Ethics

The human biological samples were sourced ethically following receipt of informed consent from each patient and their research use was in accordance with the terms of the informed consents under an IRB/EC approved protocol at participating centres.

4.2.6. Statistical Analysis

The primary endpoint of this study was to predict the presence of advanced fibrosis (F3-F4) and the presence of NASH (NAS $\geq$4, with at least one point for ballooning, lobular inflammation and steatosis). The combined cohort of 361 patients was randomly separated into approximately 2/3 (n=247) (Discovery cohort) and 1/3 (n=114) (Validation cohort) for collagen fragment biomarker investigation and validation. Similarly, a cohort of 196 patients was randomly separated into approximately 2/3 discovery cohort (n=109) and 1/3 (n=39) validation cohort for interrogation of the additional biomarkers, LOXL2, FPA and VWF. Continuous variables were compared using the t test and categorical variables using Chi-square test. The Kruskal-Wallis test was used to perform comparisons between mean marker levels followed by Dunn's multiple comparison tests. In the discovery cohort, significant variables on univariate analysis ($p<0.05$) were included in the backward stepwise multiple logistic regression analysis to identify independent factors associated with fibrosis

and NASH. Optimal cut-offs for each significant biomarker were selected using the Youden index (J-Index) which attributes equal value to sensitivity and specify. The diagnostic accuracies of all biomarkers were determined by calculating the area under the receiver operating characteristic (ROC) curve (the c-statistic) and its 95% confidence intervals. ROC curves were also calculated for the established fibrosis diagnostic scores as outlined in section 2.2 [10, 24, 27-29]. All statistical analyses were performed using SPSS software version 24.0 (SPSS Inc, Chicago, USA) and Stata version 15.1 (STATA Corp., Texas, USA)

4.3. RESULTS

4.3.1. Characteristics of Patient Population

Table 4.1 summarizes the clinico-demographic details of the Discovery study population (n=247). 18 patients had NAFL (F0 fibrosis, minimal hepatic injury), 229 patients had NASH. No differences were observed in gender, albumin, serum lipids, C4M, C3M, LOXL2 or VWF between the groups. **Table 4.2** summarises the clinic-demographic details of the validation cohort. (n=114) 14 people had NAFL, 100 people had NASH. Complete clinical data was available on all patients. No differences were observed for gender, ALT, albumin, cholesterol, C3M, LOXL2 and VWF between the groups.

Table 4.1 Baseline Demographic and Clinical characteristics of participants Discovery cohort (n=247)

	NAFL	NASH				
	F0	**F0**	**F1**	**F2**	**F3**	**F4**	**P-Value**
Number of patients	18	26	56	59	57	31	
Age (years)	49 +/- 9	42+/-12	49+/-12	51+/-14	55+/-13	58+/-8	<0.001
Gender (male)	9 (50%)	20 (77%)	35 (63%)	35 (59%)	30 (53%)	12 (39%)	0.073
BMI (Kg/m^2)	30+/-6	28+/-4	32+/-6	33+/-6	33+/-6	37+/-6	<0.001
T2DM	3 (17%)	4 (15%)	16 (29%)	35 (59%)	39 (68%)	28 (90%)	<0.001
ALT (U/l)	44+/-29	72+/-29	69+/-49	68+/-40	77+/-46	75+/-38	<0.001
AST (U/l)	30+/-12	39+/-13	43+/-26	45+/-24	56+/-24	64+/-37	<0.001
Albumin (g/dl)	44+/-3	45+/-4	44+/-6	44+/-5	44+/-5	42+/-5	0.186
Platelets ($X10^9/l$)	252+/-88	255+/-62	230+/-62	236+/-65	227+/-83	197+/-108	0.001
Cholesterol (mg/dl)	5.7+/-1	11+/-33	5.2+/-1.5	4.9+/-1.4	8.5+/-26	4.6+/-1.2	0.058
Triglycerides (mg/dl)	5.7+/-17	2.5+/-2.3	2.2+/-2	2.1+/-2	6.4+/-29	2.3+/-1.3	0.312
Collagen PROC3(ng/ml)	9.6+/-3	14.2+/-6	12.7+/-6	17+/-11	26.5+/-18	31.5+/-18.4	<0.001
Collagen PROC6(ng/ml)	7.2+/-2	8.3+/-2	8.4+/-3	9+/-3	12.5+/-7	13.1+/-5	<0.001
PROC4 (ng/ml)	217+/-90	238+/-115	264+/-116	302+/-141	317+/-159	385+/-152	<0.001
C4M (ng/ml)	26+/-9	24+/-8	27+/-9	28+/-11	29+/-10	31+/-10	0.025
C3M (ng/ml)	10+/-4	11+/-4	11+/-3	12+/-4	12+/-5	13+/-4	0.105

LOXL2 (ng/ml)	54+/-33	48+/-45	50+/-49	55+/-42	58+/-42	53+/-37	0.809
FPA (ng/ml)	4987+/-2600	3492+/-1525	4848+/-2104	4940+/-2333	4152+/-2090	3912+/-1714	0.013
VWF (ng/ml)	12+/-8	11+/-7	11+/-6	13+/-11	20+/-32	14+/-7	0.498
Fibrosis Stage (0/1/2/3/4)	18/0/0/0/0	26/0/0/0/0	0/56/0/0/0	0/0/59/0/0	0/0/0/57/0	0/0/0/0/31	<0.001
Steatosis 0/1/2/3	0/16/2/0	0/2/12/12	0/23/23/10	0/19/23/17	2/10/21/24	0/13/14/4	<0.001
Ballooning 0/1/2	16/2/0	16/5/5	14/29/13	11/30/18	3/22/32	2/15/14	<0.001
Lobular Inflammation 0/1/2/3	10/8/0/0	3/16/7/0	4/36/16/0	3/27/26/3	0/14/32/11	0/3/12/16	<0.001
NAS	2+/-0.5	4+/-1	4+/-1	4+/-2	6+/-2	5+/-1	<0.001
FIB4	1.04+/-0.54	0.89+/-0.62	1.29+/-0.83	1.40+/-0.86	1.84+/-1.14	2.68+/-1.43	<0.001
AAR	0.4+/-0.21	0.60+/-0.26	0.73+/-0.33	0.71+/-0.21	0.93+/-0.30	0.93+/-0.37	<0.001
NAFLD Fibrosis Score	-2.23+/-1.14	-3.053+/-1.296	-1.676+/-1.485	-1.321+/-1.521	-0.836+/-1.784	0.579+/-1.987	<0.001
APRI	0.40+/-0.21	0.50+/-0.3	0.61+/-0.5	0.61+/-0.39	0.80+/-0.41	1.10+/-0.58	<0.001
ADAPT Score	5+/-0.9	4.8+/-1.2	5.2+/-1.1	6.2+/-1.7	7.65+/-2.1	9.0+/-2.2	<0.001
BARD Score	2+/-1	1+/-1	2+/-1	*2*+/-1	*2*+/- 1	*3*+/-1	*<0.001*
Centrally Reviewed Biopsies	10 (56%)	15 (58%)	29 (52%)	39 (66%)	44 (77%)	28 (90%)	0.004

^The table shows the mean ± SD for continuous variables, number (%) for binary variables, and number per group for categorical variables NAS= NAFLD Activity Score Chi-Square test was used for categorical variables. Kruskal-Wallis was used for all other variables BMI= Body mass index; T2DM= Type 2 Diabetes Mellitus; ALT = Alanine Aminotransferase; AST= Aspartate aminotransferase; NAS = NAFLD activity score; FIB4= Fibrosis-4 Index; AAR= AST to ALT ratio; APRI= AST to platelet ratio index. ADAPT = Algorithm including Age, Diabetes, PROC3 and platelet count. BARD = BMI, AST/ALT ratio, Diabetes

'NAFL' was defined as steatosis only, or steatosis with mild inflammation without hepatocyte ballooning degeneration

Table 4.2 Baseline Demographic and Clinical characteristics of participants Validation cohort (n=114)							
	NAFL	**NASH**					
	F0	**F0**	**F1**	**F2**	**F3**	**F4**	**P-Value**
Number of patients	14	10	19	23	29	19	
Age (years)	54 +/- 10	47+/-14	43+/-12	47+/-14	58+/-10	58+/-9	<0.001
Gender (male)	11 (79%)	7 (70%)	11 (58%)	13 (57%)	20 (69%)	9 (47%)	0.458
BMI (Kg/m^2)	29+/-5	29+/-6	30+/-6	31+/-5	36+/-8	35+/-5	<0.001
T2DM	3 (21%)	3 (30%)	6 (32%)	7 (30%)	19 (66%)	17 (90%)	<0.001
ALT (U/l)	57+/-39	56+/-30	76+/-39	70+/-41	66+/-35	67+/-45	0.392
AST (U/l)	33+/-16	34+/-13	53+/-27	44+/-27	52+/-25	49+/-35	0.002
Albumin (g/dl)	44+/-5	45+/-4	44+/-4	46+/-3	44+/-3	42+/-4	0.057
Platelets (X10^9/l)	238+/-63	221+/-57	258+/-66	243+/-53	205+/-57	182+/-52	0.002
Cholesterol (mg/dl)	14+/-32	5+/-1	5.5+/-1.6	5.6+/-2	4.7+/-1.1	5+/-1.1	0.369
Triglycerides (mg/dl)	1.5+/-0.5	1.9+/-2.2	2.8 +/-4.7	2.7+/-1.7	9.6+/-37.4	2.0+/-0.7	0.032
Collagen PROC3(ng/ml)	11.6+/-5.2	10.9+/-4.6	17.8+/-15.7	14.6+/-4.3	25.6+/-17.2	24.5+/-15.8	<0.001
Collagen PROC6(ng/ml)	9.6+/-6.4	7.3+/-2.2	8.1+/-2.9	10.2+/-4	10.4+/-2.9	10.9+/-3.7	0.006
PROC4 (ng/ml)	255+/-137	208+/-65	224+/-86	303+/-143	297+/-96	356+/-234	0.041
C4M (ng/ml)	25+/-8	22+/-7	25+/-7	28+/-8.4	29+/-11	30+/-15	0.260
C3M (ng/ml)	12+/-6	10+/-3	6.8+/-2.8	12.6+/-4	11.7+/-3.4	12.5+/7.6-	0.235

LOXL2 (ng/ml)	44+/-30	59+/-47	13+/-5.2	86+/-95	45+/-29	60+/-55	0.353
FPA (ng/ml)	4030+/-1776	3935+/-1182	4799+/-1737	4525+/-2199	4361+/-2009	4839+/-1695	0.701
VWF (ng/ml)	7.7+/-3.3	10.1+/-6	10.8+/-4.11	13.9+/-7.4	17.9+/-5.6	21+/-15.7	<0.001
Fibrosis Stage (0/1/2/3/4)	14/0/0/0/0	10/0/0/0/0	0/19/0/0/0	0/0/23/0/0	0/0/0/29/0	0/0/0/0/19	<0.001
Steatosis 0/1/2/3	1/12/1/0	0/3/3/4	1/5/8/5	0/4/9/10	0/7/14/8	0/6/9/4	0.007
Ballooning 0/1/2	13/1/0	6/1/3	3/11/5	2/17/4	1/12/16	1/6/6	<0.001
Lobular Inflammation 0/1/2/3	6/8/0/0	2/6/2/0	0/16/2/1	0/15/8	1/8/17/3	1/8/8/2	<0.001
NAS	2+/-0.5	4+/-0.4	4+/-2	5+/-1	5+/-1	5+/-2	<0.001
FIB4	1.10+/-0.54	1.12+/-0.60	1.21+/-0.89	1.15+/-0.65	2.09+/-1.39	2.51+/-1.19	<0.001
AAR	0.65+/-0.19	0.74+/-0.44	0.82+/-0.53	0.74+/-0.35	0.85+/-0.25	0.99+/-0.31	0.007
NAFLD Fibrosis Score	-2.128+/-1.530	-1.955+/-0.968	0.647+/-0.371	-2.15+/-1.68	-0.13+/-1.14	0.610+/-1.2981	<0.001
APRI	0.42+/-0.18	0.47+/-0.18	0.65+/-0.37	0.67+/-0.43	0.87+/-0.75	0.1+/-0.54	<0.001
ADAPT Score	5.2+/-1.3	4.8+/-1.3	5.3+/-2	5.4+/-1.1	7.9+/-2.3	8.1+/-2.3	<0.001
BARD Score	1+/-1	1+/-1	2+/-1	*2*+/-1	*3*+/-1	*3*+/-1	*<0.001*
Centrally Reviewed	11 (79%)	4 (40%)	5 (26%)	14 (61%)	23 (79%)	15 (79%)	0.002

^The table shows the mean ± SD for continuous variables, number (%) for binary variables, and number per group for categorical variables NAS= NAFLD Activity Score Chi-Square test was used for categorical variables. Kruskal-Wallis was used for all other variables BMI= Body mass index; T2DM= Type 2 Diabetes Mellitus; ALT = Alanine Aminotransferase; AST= Aspartate aminotransferase; NAS = NAFLD activity score; FIB4= Fibrosis-4 Index; AAR= AST to ALT ratio; APRI= AST to platelet ratio index. ADAPT = Algorithm including Age, Diabetes, PROC3 and platelet count. BARD = BMI, AST/ALT ratio, Diabetes
NAFL was defined as steatosis only, or steatosis with mild inflammation without hepatocyte ballooning degeneration

4.3.2. Preliminary exploration serum collagen fragment levels

4.3.2.1. Serum collagen fragment level cross-correlation

Serum collagen fragment level cross-correlation was assessed in the total cohort (n=361) **(Table 4.3).** All correlations were statistically significant (p <0.0001). Levels of PROC3 and PROC6 were strongly correlated (Rs = 0.612) **Figure 4.2**. C3M, C4M and PROC4 levels were also strongly correlated.

Table 4.3. Cross Correlation of serum collagen biomarkers (n=361)

Biomarkers	Spearmann Rank Correlation Co-efficient (r_s)	P-value
C3M/C4M	0.835	<0.0001
C3M/PROC3	0.319	<0.0001
C3M/PROC4	0.824	<0.0001
C3M/PROC6	0.353	<0.0001
C4M/PROC3	0.309	<0.0001
C4M/PROC4	0.862	<0.0001
C4M/PROC6	0.383	<0.0001
PROC3/PROC4	0.344	<0.0001
PROC3/PROC6	0.612	<0.0001
PROC4/PROC6	0.461	<0.0001

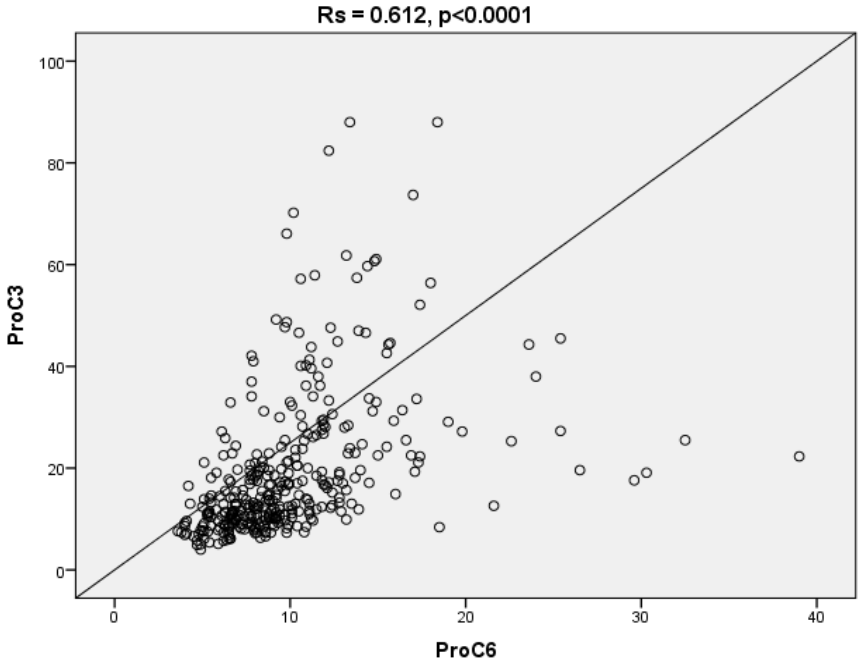


Figure 4.2. PROC3 and PROC6 cross correlation

4.3.3. Serum collagen fragment level and NAFLD histological stage

4.3.3.1. Fibrosis

Mean serum levels of all collagen fragments increased incrementally with increasing fibrosis stage. Excluding C3M, serum collagen fragment levels differences reached statistical significance between (1) all stages of fibrosis, (2) mild versus severe fibrosis and (3) NAFL versus NASH **(Table 4.4).** PROC3 levels were significantly higher between patients with mild versus severe fibrosis **Figure 4.3**. Mean PROC3 levels in the severe fibrosis group (F3-F4) were measured at 27.5ng/ml and were approximately 2-fold (47%) higher than in the mild fibrosis group (F0-F2) (14.6ng/ml). PROC6 levels in the severe fibrosis group (F3-F4) were measured at 11.9ng/ml were approximately 1.5 (29%) times higher than in the mild group (8.5ng/ml).

Table 4.4 Serum collagen biomarker level analysis (n=361) – Fibrosis stage

	NAFL	**NASH**								
	F0	**F0**	**F1**	**F2**	**F3**	**F4**	**P-Value All stages**	**P-Value F0-2 V F3-4**	***% difference mild versus severe(ng/ml)**	**P-value NAFL V NASH**
Number of patients	32	36	75	82	86	50				
PROC3	10.4 +/- 4.3	13.3 +/- 5.6	14 +/- 9	16.7 +/-9	26 +/- 18	28.9 +/- 18	<0.0001	<0.0001	47%	<0.0001
PROC4	234 +/- 112	229 +/- 104	253 +/- 110	302 +/- 141	309 +/- 141	357 +/- 200	<0.0001	<0.0001	22%	0.012
PROC6	8.3 +/-4.6	8 +/- 2.3	8.4 +/- 2.9	9.6 +/- 3.6	11.7 +/- 5.7	12 +/- 5	<0.0001	<0.0001	29%	0.001
C3M	11 +/- 5	10 +/-3	11 +/- 3	12 +/-4	12 +/-5	13 +/- 6	0.066	0.156	15%	0.262
C4M	25 +/- 9	23 +/-7	26 +/- 9	28 +/- 10	29 +/- 10	30 +/- 12	0.004	0.007	13%	0.224

*Values are mean and Standard Deviation (ng/ml). Statistical test; Kruskal-Wallis

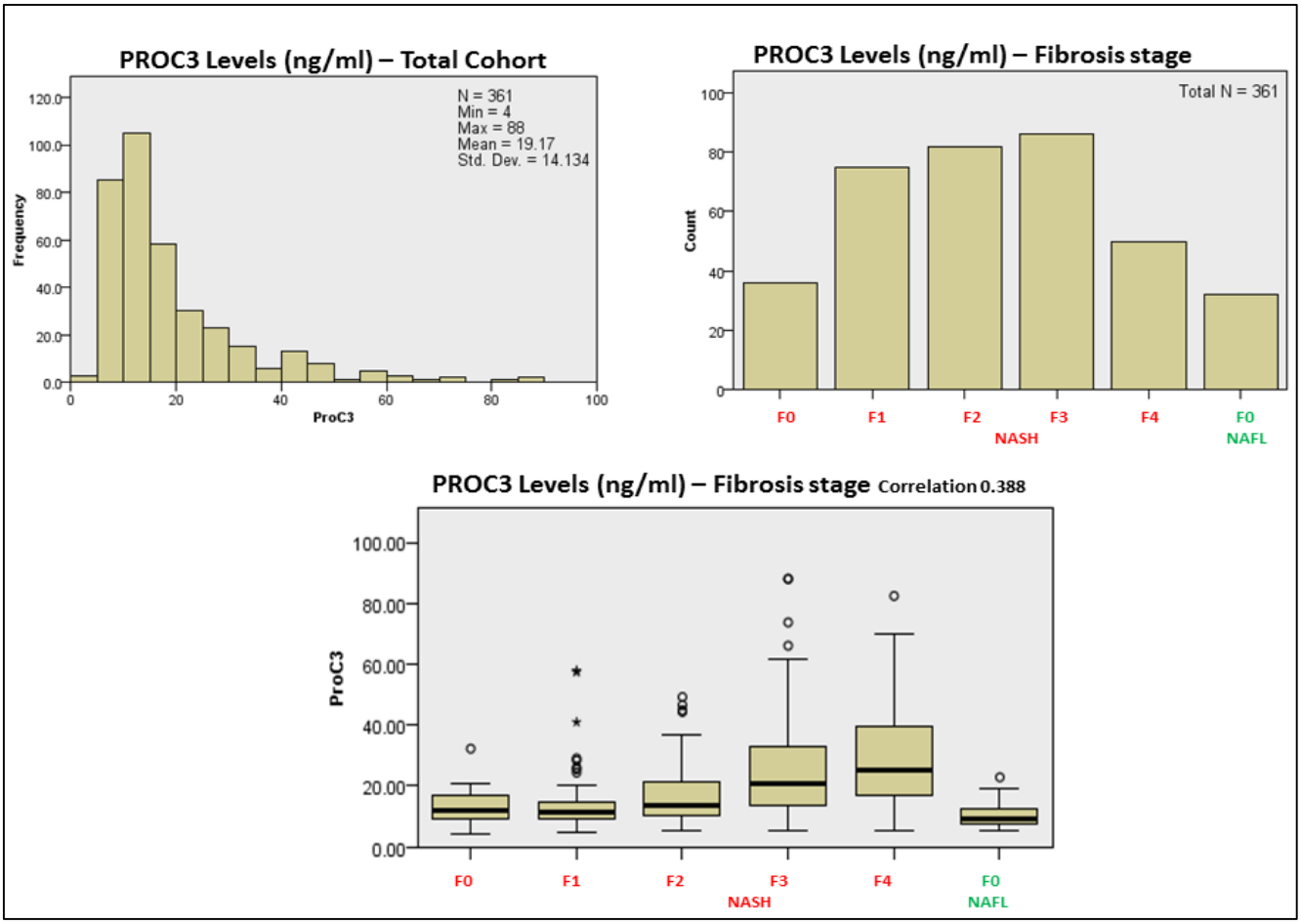


Figure 4.3. Serum PROC3 Levels

A= *Histogram PROC3 measurements****;***

B= *Number of PROC3 measurements as per fibrosis stage*

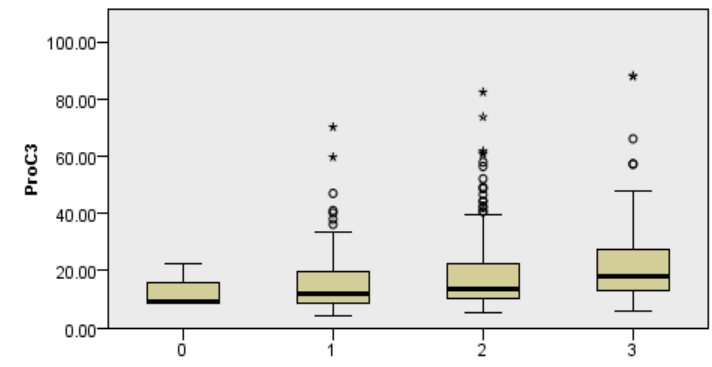
C= *Mean serum PROC3 levels as per fibrosis stage*

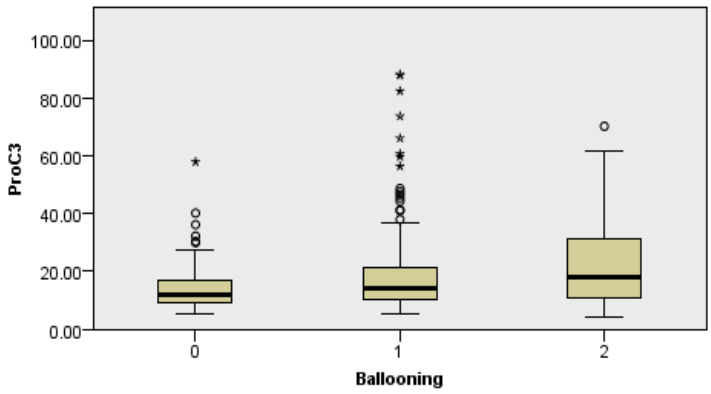
4.3.3.2. Serum collagen fragment correlation with steatohepatitis

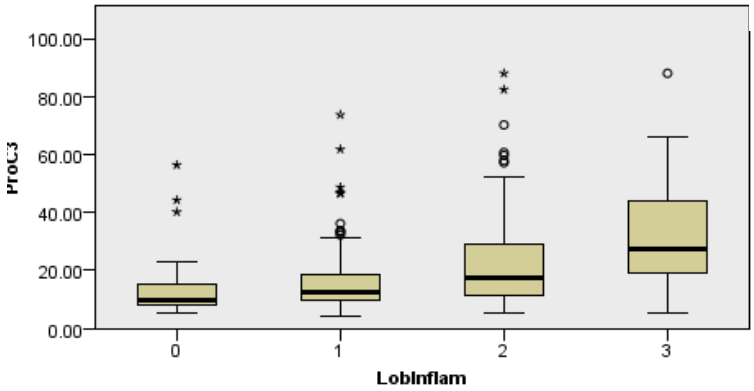
Serum collagen fragment levels did not ***correlate strongly*** with steatohepatitis components or the NAS score. PROC3 levels did statistically correlate with NASH components however the correlations were weak (Rs=<0.388) **(Table 4.5)**. Mean PROC3 levels were significantly higher in patients with steatosis, ballooning, lobular inflammation and NASH **(Table 4.6)**. Like PROC3, PROC6 levels were statistically associated with ballooning, lobular inflammation and the NAS score **(Figure 4.4)**.

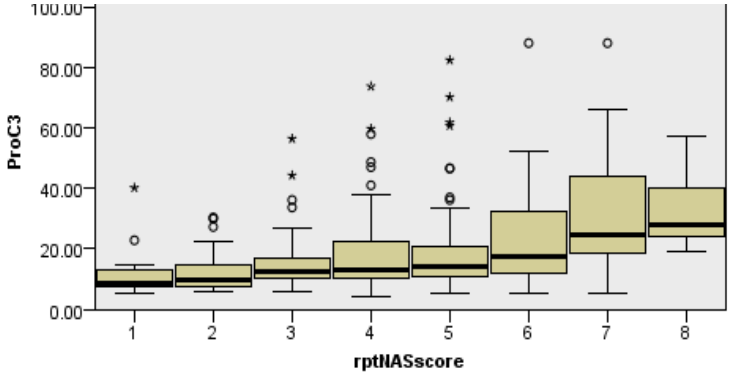
Table 4.5 Spearmann Rank Correlation Co-efficient with steatohepatitis components										
N=361	**PROC3**	**P-value**	**PROC4**	**P-value**	**PROC6**	**P-value**	**C3M**	**P-value**	**C4M**	**P-value**
Steatosis	0.261	**<0.0001**	0.101	0.055	0.018	0.743	0.088	0.095	0.092	0.081
Hepatocyte Ballooning	0.228	**<0.0001**	0.020	0.710	0.170	**0.001**	-0.20	0.702	0.040	0.452
Lobular Inflammation	0.338	**<0.0001**	0.082	0.119	0.097	**<0.0001**	0.07	0.182	0.093	0.077
NAS Score	0.388	**<0.0001**	0.094	0.077	0.169	**0.002**	0.058	0.277	0.101	0.058
*Statistical test; Spearmann Rank Correlation Co-efficient (Rs)										

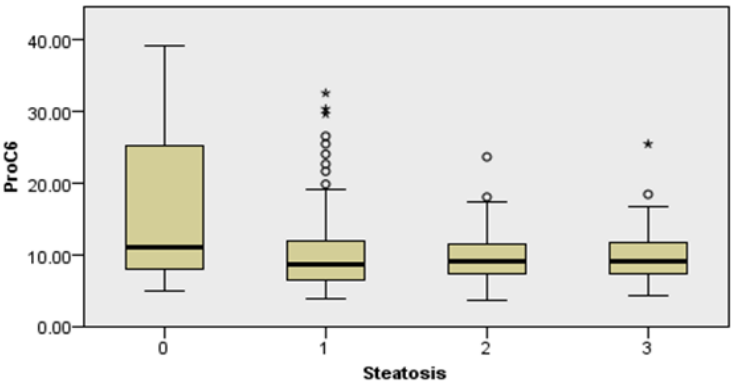
Table 4.6. Association of mean serum collagen fragment levels and steatohepatitis					
N=361	**PROC3**	**PROC4**	**PROC6**	**C3M**	**C4M**
Steatosis	<0.0001	0.189	0.688	0.146	0.354
Hepatocyte Ballooning	<0.0001	0.180	0.003	0.596	0.655
Lobular Inflammation	<0.0001	0.379	0.002	0.529	0.356
NAS Score	<0.0001	0.251	0.009	0.581	0.266
Statistical test; Kruskal Wallis					

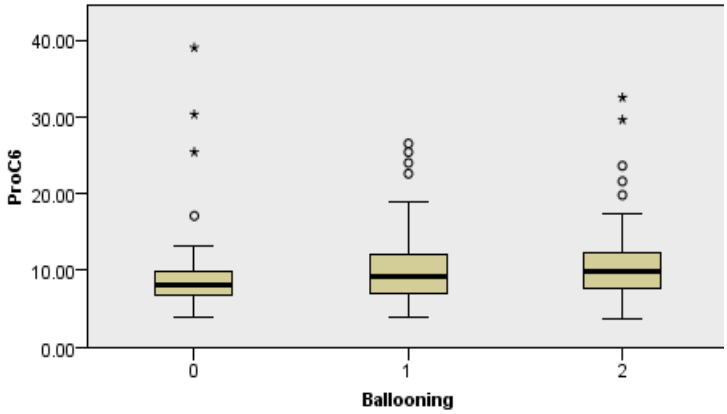


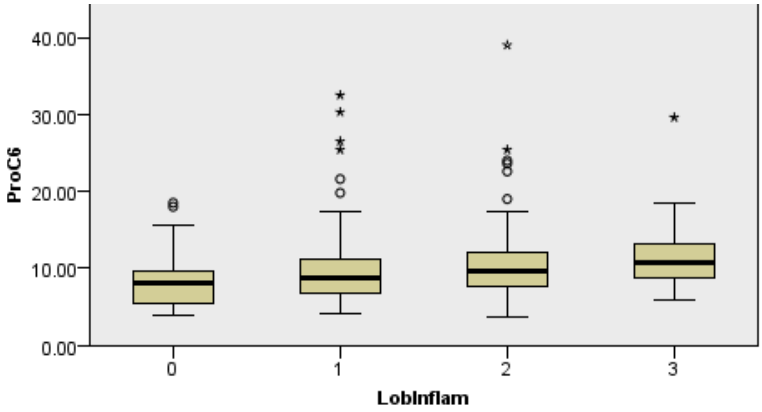












*Statistical test; Kruskal-Wallis. Refer to **Table 4.6** for p-values*

Figure 4.4 Mean serum PROC3 and PROC6 levels and NAFLD histology

4.3.4. Performance of serum collagen fragments to discriminate fibrosis stage in NAFLD

4.3.4.1. Mild versus severe fibrosis (F0-F2 versus F3-F4)

The results of univariate and multivariate analyses preformed to detect the presence of advanced fibrosis in the discovery cohort are shown in **Table 4.7**. Using backward logistic regression, only 3 collagen fragments (PROC3, PROC6 and C4M) remained predictive of advanced fibrosis. The AUROC for the three significant collagen fragments and the optimal cut-off levels for the detection of advanced fibrosis as per the Youden Index (YI) are reported in **Table 4.8** and **figure 4.5**.

Table 4.7 Univariate and multivariate analysis to detect the presence of NASH

Advanced fibrosis (F0-F2 versus F3-F4)						
N=247	**Univariate**			**Adjusted (Multivariate)**		
Variable	**Odds Ratio**	**95% CI**	**p-value**	**Odds Ratio**	**95% CI**	**p-value**
PROC3	1.100	1.07-1.134	<0.0001	1.067	1.029-1.107	<0.0001
PROC4	1.003	1.00-1.005	0.001			
PROC6	1.279	1.17-1.399	<0.0001	1.141	1.028-1.265	0.013
C3M	1.046	0.98-1.11	0.157			
C4M	1.027	1.0-1.055	0.048	0.906	0.835-0.982	0.016
Age	1.047	1.02-1.072	<0.0001	1.040	1.008-1.073	0.015
Gender	1.807	1.07-3.061	0.028			
BMI	1.081	1.04-1.130	<0.0001			
T2DM	5.556	3.09-9.992	<0.0001	3.036	1.451-6.352	0.003
ALT	1.005	0.10-1.011	0.095			
AST	1.030	1.02-1.043	<0.0001	1.020	1.005-1.035	0.007
Albumin	0.969	0.92-1.018	0.212			
Platelets	0.996	0.99-1.000	0.033			
Cholesterol	1.003	0.98-1.019	0.673			
Triglycerides	1.012	0.99-1.04	0.340			

The Youden Index (YI) was used to determine the optimal cut-off points for the detection of advanced fibrosis (F3-F4). The accuracy of PROC3 and PROC6 for the differentiation of mild versus severe fibrosis were similar at 74% and 75%, with AUROCs of 0.78 and 0.75 respectively. Combined, PROC3 and PROC6 performed marginally better with an accuracy of 79% and an AUROC of 0.79. C4M had the poorest performance with an accuracy of 61% and AUROC of 0.59. The ability of these biomarkers to discriminate specific fibrosis stages is shown in **Table 4.9.** The predictive power of each biomarker improved beyond F3 fibrosis.

Table 4.8 Diagnostic accuracy PROC3, PROC6, C4M for the detection of advanced fibrosis				
	PROC3	**PROC6**	**PROC3+PROC6**	**C4M**
AUROC	0.78	0.75	0.79	0.59
Optimal Cut off	>17.5ng/ml	>10.5ng/ml	>34.1ng/ml	>27.7ng/ml
Sensitivity	68%	62%	59%	57%
Specificity	77%	83%	89%	63%
PPV	63%	67%	76%	46%
NPV	81%	80%	80%	72%
Accuracy	74%	75%	79%	61%

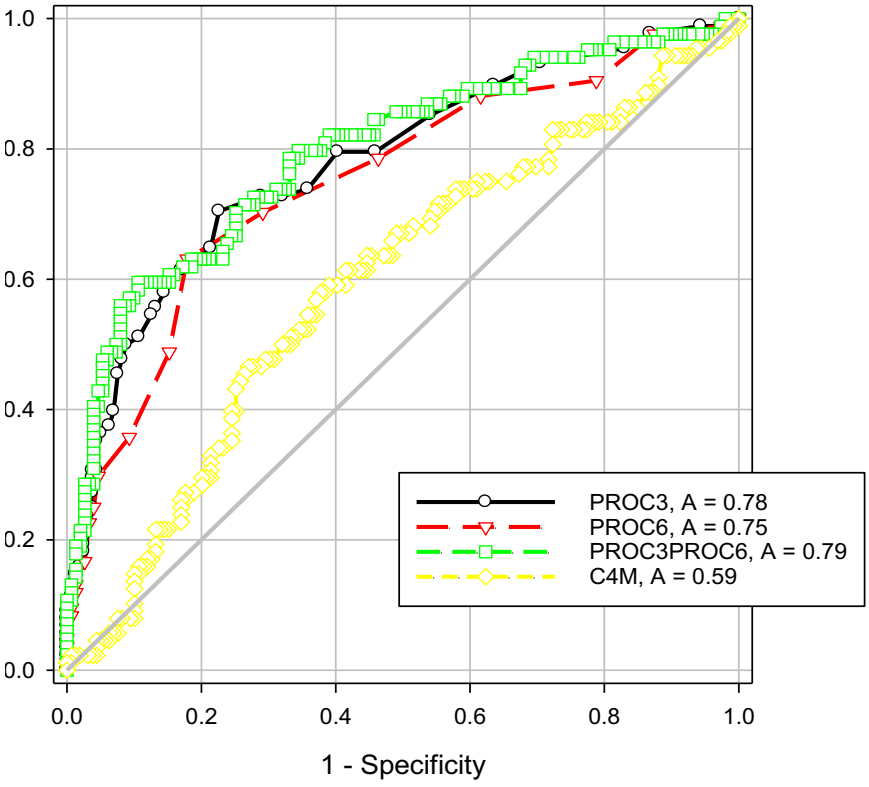


Figure 4.5. AUROC PROC3, PROC6, PROC3+PROC6, C4M for the detection of advanced fibrosis

Table 4.9. AUROC in discriminating specific fibrosis stages				
	PROC3	**PROC6**	**PROC3+PROC6**	**C4M**
AUROC				
F0	0.69	0.68	0.71	0.63
F1	0.68	0.64	0.68	0.53
F2	0.47	0.46	0.47	0.52
F3	0.70	0.68	0.71	0.54
F4	0.76	0.73	0.78	0.63

4.3.5. Serum collagen fragment levels to discriminate NASH versus NAFL

The results of univariate and multivariate analyses preformed in the discovery cohort to detect the presence of NASH are shown in **Table 4.10**. NASH was defined as NAS score ≥4. Using backward logistic regression, only PROC3 remained significantly associated with NASH. The AUROC for all collagen fragments for NASH detection is reported in **Table 4.11**. The Optimal cut-off levels for the detection of NASH was derived for all serum collagen fragments using the Youden Index in **Table 4.12.** PROC3 had the best AUROC for the detection of NASH. Optimal cut-off was derived at a level of >16.5ng/ml. At this cut-off PROC3 had a sensitivity of 54% and specificity of 78% for NASH detection.

Table 4.10. Univariate and Multivariate analysis to identify biomarkers associated with NASH						
NASH detection (NAS≥4)						
N=247	**Univariate**			**Adjusted (Multivariate)**		
Variable	**Odds Ratio**	**95% CI**	**p-value**	**Odds Ratio**	**95% CI**	**p-value**
PROC3	1.081	1.041-1.122	<0.0001	1.059	1.019-1.100	0.003
PROC4	1.000	0.998-1.002	0.788			
PROC6	1.018	0.957-1.083	0.568			
C3M	1.010	0.943-1.081	0.785			
C4M	1.009	0.979-1.040	0.545			
Age	0.993	0.972-1.015	0.523			
Gender	0.722	0.415-1.257	0.250			
BMI	1.030	0.983-1.079	0.212			
T2DM	2.039	1.162-3.578	0.013	2.045	1.078-3.880	0.029
ALT	1.031	1.019-1.043	<0.0001	1.030	1.017-1.043	<0.0001
AST	1.062	1.038-1.086	<0.0001			
Albumin	1.050	0.996-1.107	0.071			
Platelets	1.000	0.997-1.004	0.966			

Table 4.11. Synopsis of collagen fragment level diagnostic accuracy for the detection of NASH versus NAFL					
N=247	**PROC3**	**PROC4**	**PROC6**	**C3M**	**C4M**
AUROC	0.69	0.53	0.56	0.54	0.54
P-Value	< 0.0001	0.46	0.14	0.31	0.31
Cut -off	>16.5ng/ml	>270ng/ml	>7.5ng/ml	>9.1ng/ml	>20.35ng/ml
Sensitivity	54%	48%	73%	73%	80%
Specificity	78%	63%	38%	38%	32%
PPV	85%	75%	75%	73%	74%
NPV	42%	34%	36%	37%	40%
Accuracy	61%	52%	63%	62%	66%

4.3.6. Validation of Collagen fragments for the detection of advanced fibrosis and NASH

The performance of PROC3, PROC6 and C4M to identify patients with advanced fibrosis was validated in an independent cohort of 114 subjects **Table 4.12**. The clinico-demographic details of this population are outlined in **Table 4.2.** PROC3 performed superior to the other collagen fragments with a sensitivity of 58%, specificity of 80% and accuracy of 71% at a cut-off level of >17.5ng/ml.

Table 4.12 Diagnostic accuracy PROC3, PROC6, C4M for the detection of advanced fibrosis				
N=114	**PROC3**	**PROC6**	**PROC3+PROC6**	**C4M**
AUROC	0.73	0.68	0.74	0.57
Optimal Cut off for the detection of advanced fibrosis	>17.5ng/ml	>10.5ng/ml	>34.1ng/ml	>27.7ng/ml
Sensitivity	58%	49%	38%	46%
Specificity	80%	77%	86%	70%
PPV	68%	61%	67%	52%
NPV	73%	67%	65%	64%
Accuracy	71%	65%	66%	60%

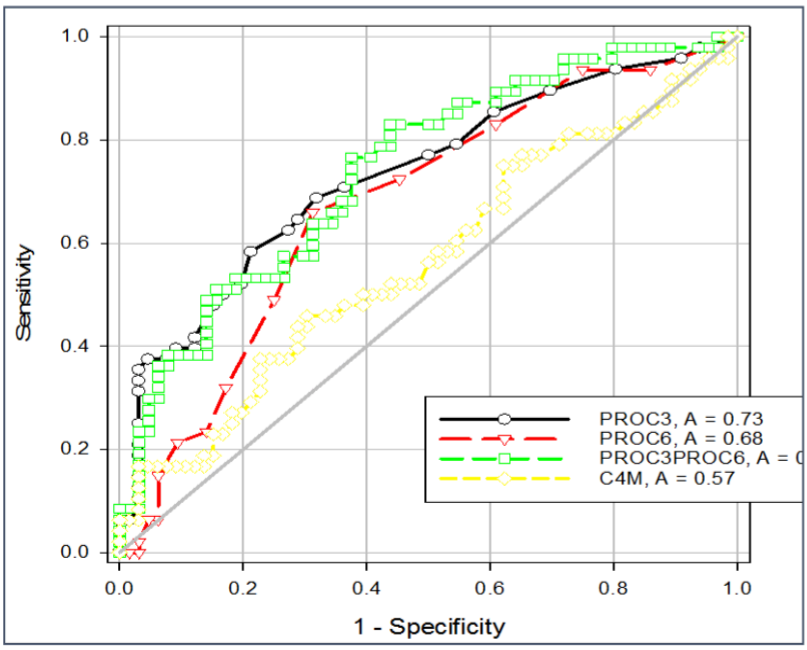


Figure 4.6 Biomarker AUROC for advanced fibrosis detection, validation cohort (n=114)

The diagnostic accuracy of serum collagen fragment levels to identify patients with NASH was evaluated in the validation cohort (n=114) **Table 4.13.** Results were similar to those obtained in the Discovery cohort. PROC3 demonstrated the best and only statistically significant AUROC of 0.63 with an accuracy of 53% and modest values for sensitivity (46%), specificity (71%) for NASH detection.

Table 4.13. Diagnostic accuracies collagen fragments in validation cohort for NASH Detection					
N=114	**PROC3**	**PROC4**	**PROC6**	**C3M**	**C4M**
AUROC	0.63	0.54	0.57	0.49	0.55
P-Value	0.03	0.48	0.29	0.92	0.43
Cut -off	>16.5ng/ml	>270ng/ml	>7.5ng/ml	>9.1ng/ml	>20.35ng/ml
Sensitivity	46	45	74	63	75
Specificity	71	65	42	39	35
PPV	80	77	76	72	75
NPV	34	31	39	29	35
Accuracy	53	50	65	56	64

4.3.7. Diagnostic accuracy comparison; Serum collagen fragments versus validated fibrosis indices

The diagnostic accuracy of routinely used fibrosis indices in current clinical use were assessed in the total cohort and compared to the predictive power of the collagen fragments investigated in this study. **Table 4.14** Complete data was available for comparison in a cohort of 346 subjects. As a single biomarker, PROC3 had the best AUROC (0.77) for the detection of advanced fibrosis, with a sensitivity, specificity, PPV, NPV and accuracy of 66%, 77%, 64%. 79% and 73% respectively. It performed similarly to previously validated fibrosis indices.

Table 4.14. Diagnostic accuracy comparison; Serum collagen fragments versus validated fibrosis indices in total cohort for the detection of advanced fibrosis (F3-4) (n=346)										
N=346	**AAR**	**APRI**	**BARD**	**NFS**	**FIB4**	**PROC3**	**PROC4**	**PROC6**	**C3M**	**C4M**
AUROC	0.68	0.75	0.73	0.80	0.78	0.76	0.63	0.73	0.55	0.59
Cut -off	>0.8	>1.5	≥2	>0.676	>2.67	>17.5ng/ml	>266ng/ml	>10.5ng/ml	>7.7ng/ml	>22.7ng/ml
Sensitivity	54%	11%	82%	31%	22%	66%	61%	57%	83%	53%
Specificity	68%	96%	51%	95%	94%	77%	64%	81%	16%	66%
PPV	51%	63%	50%	79%	71%	64%	51%	64%	38%	48%
NPV	71%	64%	83%	69%	67%	79%	73%	76%	61%	69%
Accuracy	63%	64%	63%	71%	67%	73%	63%	72%	41%	61%

4.3.8. Sub-study; Investigation LOXL2, FPA, VWF for the detection of advanced fibrosis

Additional biomarker data was available in a cohort of 196 subjects. The clinico-demographic details of this subset of patients (n=148) subdivided into a Discovery (n=109) and validation cohort (n=39) are shown in **Table 4.15**. Differences were observed between the Discovery and validation cohorts in terms of NAS score. Complete clinical data was available on all subjects.

Table 4.15 Baseline Demographic and Clinical characteristics of participants				**Discovery vs. Validation Cohort**
Variable	**All Patients (n=148)**	**Discovery Cohort (n=109)**	**Validation Group (n=39)**	**P-Value**
Age (years)	50+/-13	50+/-13	51 +/- 12	*0.665*
Gender (male)	88 (60%)	63 (58%)	25 (64%)	*0.491*
BMI (Kg/m^2)	32+/-6	32+/-6	32+/-6	*0.318*
T2DM	69 (47%)	51 (47%)	18 (46%)	*0.946*
ALT (U/l)	72+/-46	73+/-47	68+/-43	*0.461*
AST (U/l)	50+/-31	48+/-29	54+/-37	*0.910*
Albumin (g/dl)	43+/-5	43+/-6	43+/-4	*0.993*
Platelets ($X10^9/l$)	235+/-72	239+/-76	224+/-60	*0.225*
Cholesterol (mg/dl)	8.3+/-23	8.3+/-24	8.0+/-19	*0.410*
Triglycerides (mg/dl)	5.5+/-25	4.8+/-22	7.5+/-33	*0.432*
Collagen PRO-C3(ng/ml)	20+/-15	20+/-16	21+/-14	*0.592*
Collagen PRO-C6(ng/ml)	10+/-5	10+/-5	10+/-4	*0.606*
PRO-C4 (ng/ml)	275+/-124	276+/-129	272+/-110	*0.901*
C4M (ng/ml)	27+/-10	27+/-10	27+/-11	*0.908*
C3M (ng/ml)	12+/-4	12+/-4	12+/-5	*0.870*
LOXL2 ng/ml	53+/-44	54+/-42	52+/-50	*0.601*
FPA ng/ml	3982+/-1880	4015+/-1973	3889+/-1612	*0.130*
VWF ng/ml	14+/-17	14+/-20	13+/-8	*0.665*
Fibrosis Stage (0/1/2/3/4)	42/26/34/29/17	27/22/28/19/13	15/4/6/10/4	*0.207*
Steatosis 0/1/2/3	1/56/45/46	0/39/35/35	1/17/10/11	*0.285*
Ballooning 0/1/2	49/56/43	36/45/28	13/11/15	*0.233*
Lobular Inflammation 0/1/2/3	19/71/49/9	14/52/38/5	5/19/11/4	*0.592*

NAS	4+/-2	4+/-2	4+/-2	*0.036*
Centrally Reviewed Biopsies	111 (75%)	82 (75%)	29 (74%)	0.914

^The table shows the mean ± SD for continuous variables, number (%) for binary variables, and number per group for categorical variables NAS= NAFLD Activity Score
T-Test/Mann Whitney was used to test for significant differences within continuous variables and Chi-Square test was used for categorical variables.
BMI= Body mass index; T2DM= Type 2 Diabetes Mellitus; ALT = Alanine Aminotransferase; AST= Aspartate aminotransferase; NAS = NAFLD activity score; FIB4= Fibrosis-4 Index; AAR= AST to ALT ratio; APRI= AST to platelet ratio index. ADAPT = Algorithm including Age, Diabetes, PRO-C3 and platelet count. BARD = BMI, AST/ALT ratio, Diabetes

4.3.8.1. Correlation of LOXL2, FPA and VWF levels with fibrosis and steatohepatitis

Spearmann Rank correlation co-efficient values demonstrate weak (Rs<0.5) and non-statistically significant associations with steatohepatitis and fibrosis in comparison with collagen biomarker fragments (PROC3, PROC6, C4M) **Table 4.16**. The lack of association of LOXL2, FPA and VWF was again confirmed by the lack of association with mean biomarker level and histology and NAS score explored by Kruskal-Wallis testing **Figure 4.7/4.8.**

Table 4.16 Correlation of LOXL2, FPA and VWF levels with steatohepatitis and fibrosis stage

	PROC 3	P-value	PROC 6	P-value	C4M	P-value	LOXL2	P-value	FPA	P-value	VWF	P-value
Steatosis	0.246	0.003	0.47	0.574	0.1	0.227	0.078	0.344	-0.061	0.458	-0.002	0.977
Hepatocyte Ballooning	0.361	<0.0001	0.247	0.003	0.054	0.516	-0.042	0.611	0	0.997	0.069	0.403
Lobular Inflammation	0.439	<0.0001	0.224	0.006	0.105	0.203	0.059	0.476	-0.127	0.125	0.071	0.939
NAS Score	0.492	<0.0001	0.435	0.026	0.134	0.183	0.059	0.478	-0.083	0.318	0.075	0.367
Fibrosis	0.505	<0.0001	0.183	<0.0001	0.110	0.106	0.079	0.341	0.093	0.259	0.201	0.014

*Spearmann rank correlation co-efficient

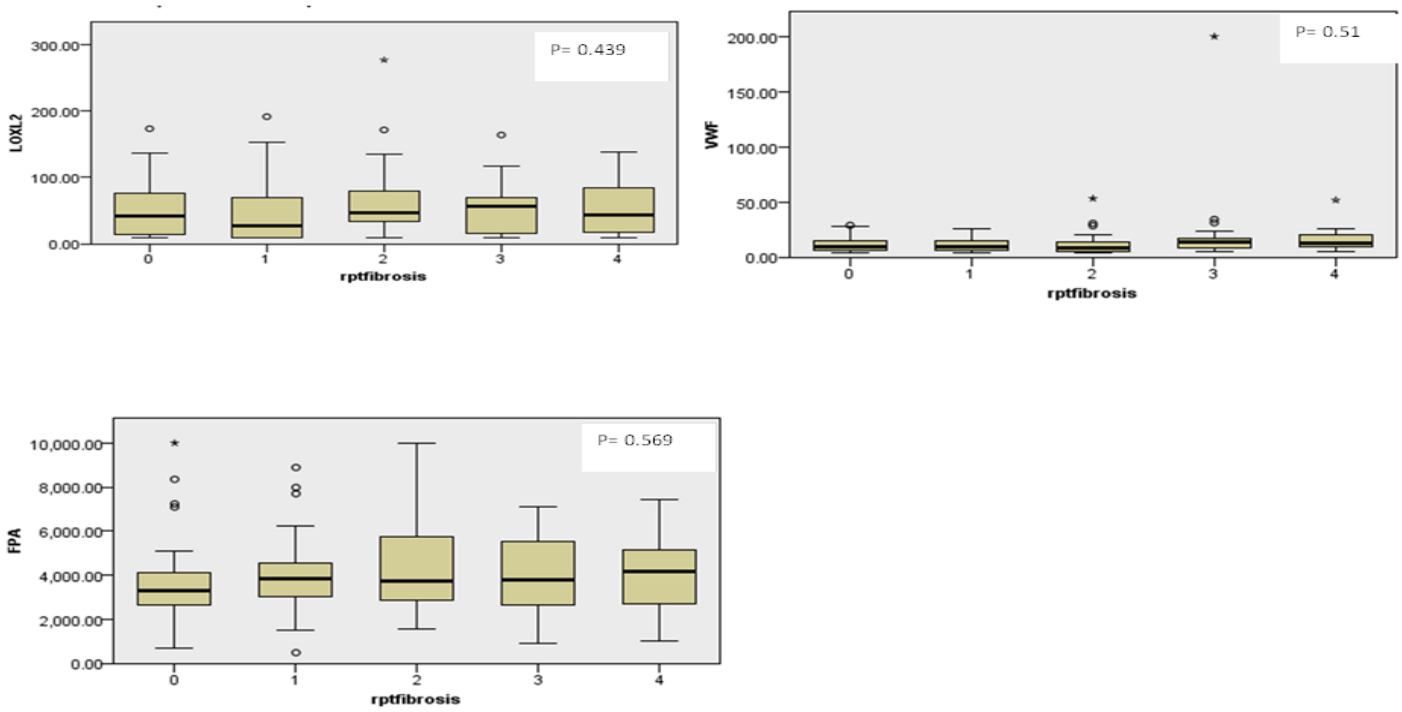


Figure 4.7 Association of LOXL2, FPA and VWF with fibrosis stage

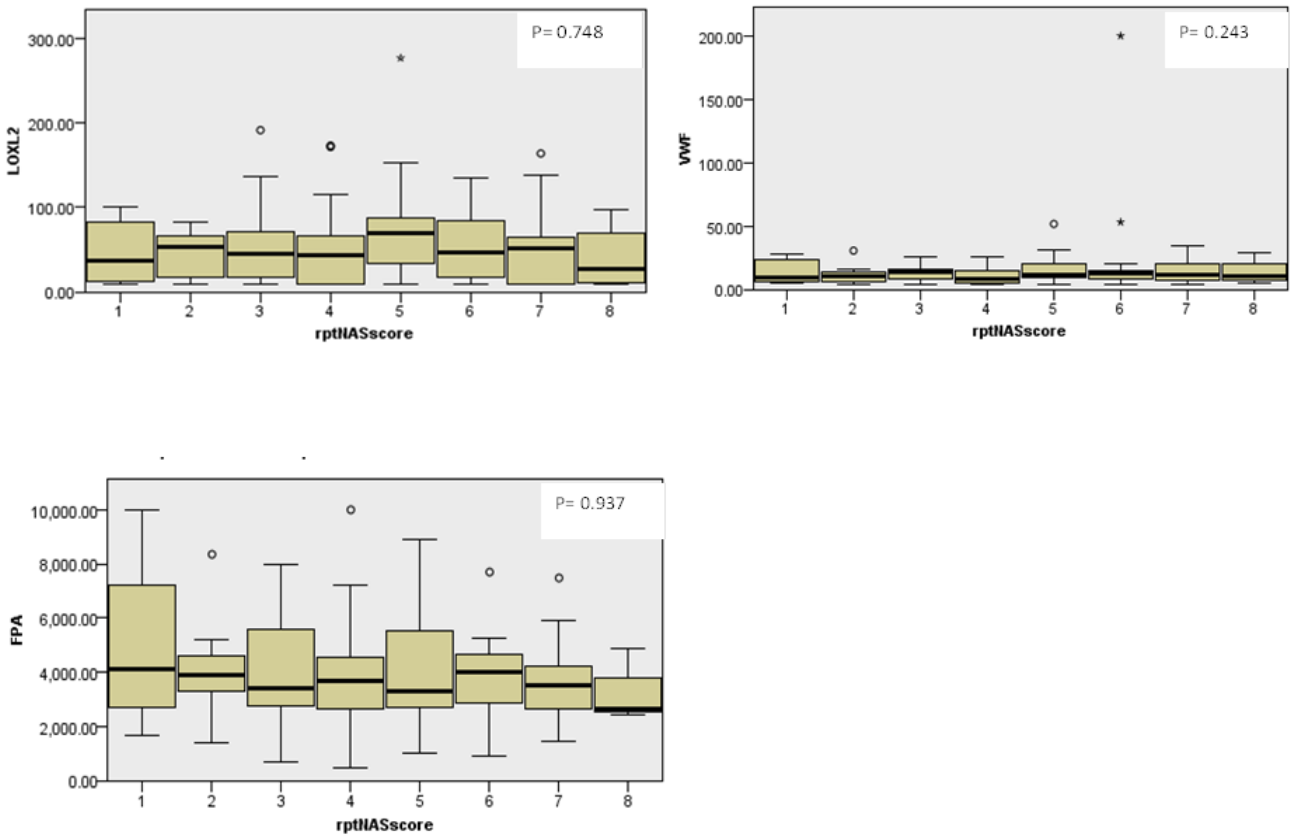


Figure 4.8. Association of LOXL2, FPA and VWF with NAS score

4.3.8.2. Performance of LOXL2, VWF and FPA to discriminate fibrosis stage in NAFLD (Discovery cohort n=109)

4.3.8.2.1. Mild versus severe fibrosis (F0-F2 versus F3-F4)

The results of univariate and multivariate analyses preformed in the discovery cohort to detect advanced fibrosis are shown in **Table 4.17**. Using backward logistic regression, only 1 biomarker (vWF) remained significantly associated with advanced fibrosis. The AUROC for vWF **(Figure 4.9)** and optimal cut-off is reported in **Table 4.18**. Data on PROC3, PROC6 and C4M have been included for comparison. The AUROC for vWF is below the accepted value for clinical utility at 0.63. Again, PROC3 and PROC6 are observed to have superior diagnostic accuracies with AUROC values of 0.75 and 0.79 respectively.

Table 4.17 Univariate and multivariate analysis to identify biomarkers associated with advanced fibrosis in Discovery cohort (n=109)

Advanced fibrosis (F0-F2 versus F3-F4)						
N=109	**Univariate**			**Adjusted (Multivariate)**		
Variable	**Odds Ratio**	**95% CI**	**p-value**	**Odds Ratio**	**95% CI**	**p-value**
LOXL2	1.001	0.993-1.009	0.834			
FPA	1.000	1.000-1.000	0.771			
VWF	1.050	1.005-1.097	0.028	1.056	1.003-1.111	0.039
Age	1.057	1.024-1.091	0.001	1.044	1.006-1.083	0.021
Gender	2	0.986-4.055	0.055			
BMI	1.116	1.047-1.189	0.001			
T2DM	5.424	2.499-11.770	<0.0001	4.249	1.752-10.303	0.001
ALT	1.005	0.998-1.013	0.152			
AST	1.018	1.006-1.030	0.004	1.014	1.002-1.027	0.026
Albumin	0.978	0.917-1.044	0.503			
Platelets	0.995	0.989-1.001	0.083			
Cholesterol	1.001	0.987-1.016	0.849			
Triglycerides	1.015	0.994-1.036	0.159			

Table 4.18 Synopsis of diagnostic accuracy of VWF to detect advanced fibrosis				
N=109	**PROC3**	**PROC6**	**VWF**	**C4M**
AUROC	0.75	0.79	0.63	0.51
Optimal Cut off for the detection of advanced fibrosis	>17.5ng/ml	>10.5ng/ml	>14.5ng/ml	>27.7ng/ml
Sensitivity	69%	66%	50%	38%
Specificity	70%	79%	74%	62%
PPV	49%	57%	44%	29%
NPV	84%	85%	78%	71%
Accuracy	70%	75%	67%	55%

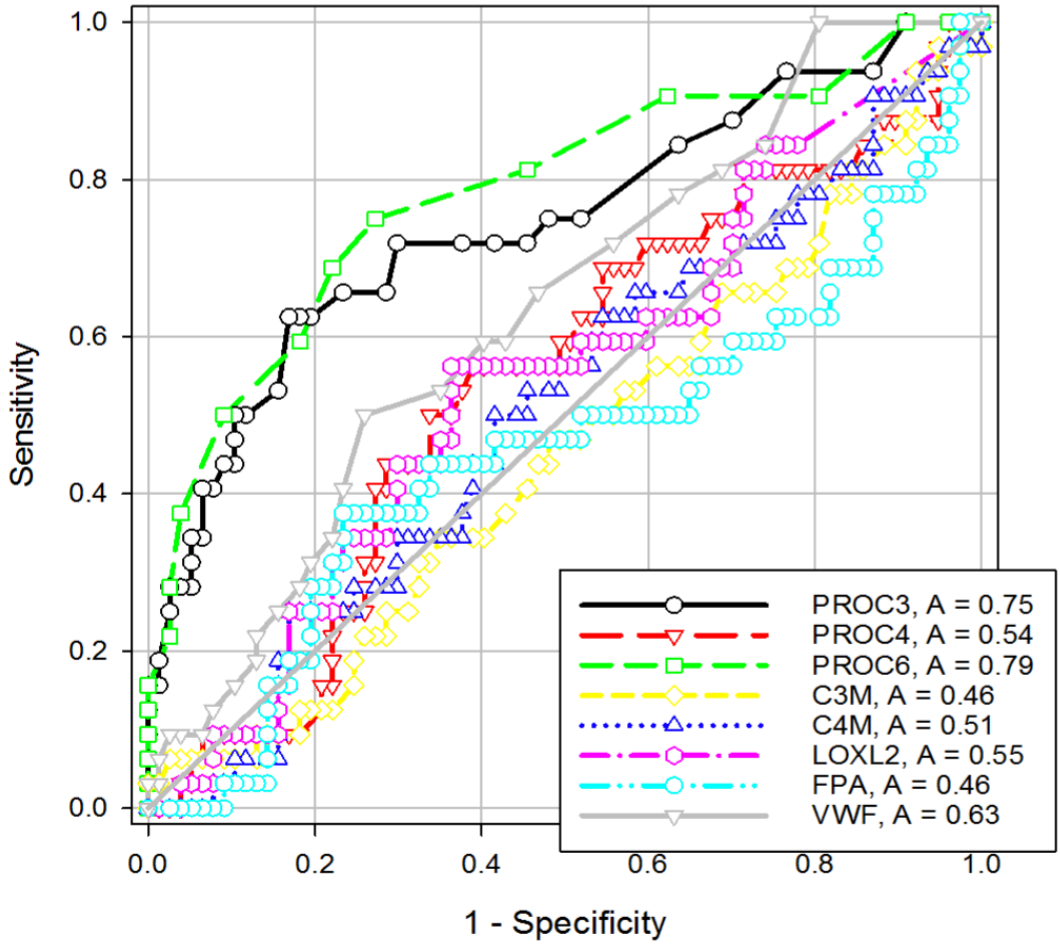


Figure 4. 9. AUROC collagen fragments and LOXL2, FPA and VWF for detection of advanced fibrosis

4.3.9. Performance of LOXL2, VWF and FPA to discriminate NASH versus NAFL

The results of univariate analysis preformed in the discovery cohort to detect NASH is shown in ***table 4.19***. None of the additional biomarkers were significant on univariate analysis for the detection of advanced NASH. The analysis was arrested at this point.

Table 4.19 Detection of NASH versus NAFL n=109			
	Univariate		
Variable	**Odds Ratio**	**95% CI**	**p-value**
LOXL2	1.005	0.995-1.016	0.321
FPA	1.000	1-1	0.219
VWF	1.003	0.980-1.027	0.775
Age	0.983	0.951-1.016	0.303
Gender	0.708	0.311-1.615	0.412
BMI	0.990	0.923-1.062	0.780
T2DM	2.167	0.923-5.089	0.076
ALT	1.034	1.016-1.052	<0.0001
AST	1.076	1.035-1.118	<0.0001
Albumin	1.052	0.976-1.133	0.184
Platelets	0.999	0.994-1.004	0.730

Table 4.19 Univariate analysis for the detection of NASH

4.3.10. Validation of Collagen fragments and VWF for advanced fibrosis detection

The performance of VWF to identify patients with advanced fibrosis was evaluated in an independent validation cohort of 39 subjects **Table 4.20**. The clinico-demographic details of this population are outlined in **Table 4.15.** The diagnostic accuracies of collagen fragments (PROC3, PROC6 and C4M) were included for comparison**.** PROC3 performed superior to the other biomarkers with a sensitivity of 79%, specificity of 80% and accuracy of 79% at a cut-off level of >17.5ng/ml.

Table 4.20. Diagnostic accuracy PROC3, PROC6, C4M and VWF for the detection of advanced fibrosis				
N=39	**PROC3**	**PROC6**	**C4M**	**VWF**
AUROC	0.85	0.69	0.52	0.75
Optimal Cut off	>17.5ng/ml	>10.5ng/ml	>27.7ng/ml	>14.5ng/ml
Sensitivity	79%	43%	36%	29%
Specificity	80%	68%	68%	84%
PPV	69%	43%	38%	50%
NPV	87%	68%	65%	68%
Accuracy	79%	59%	56%	64%

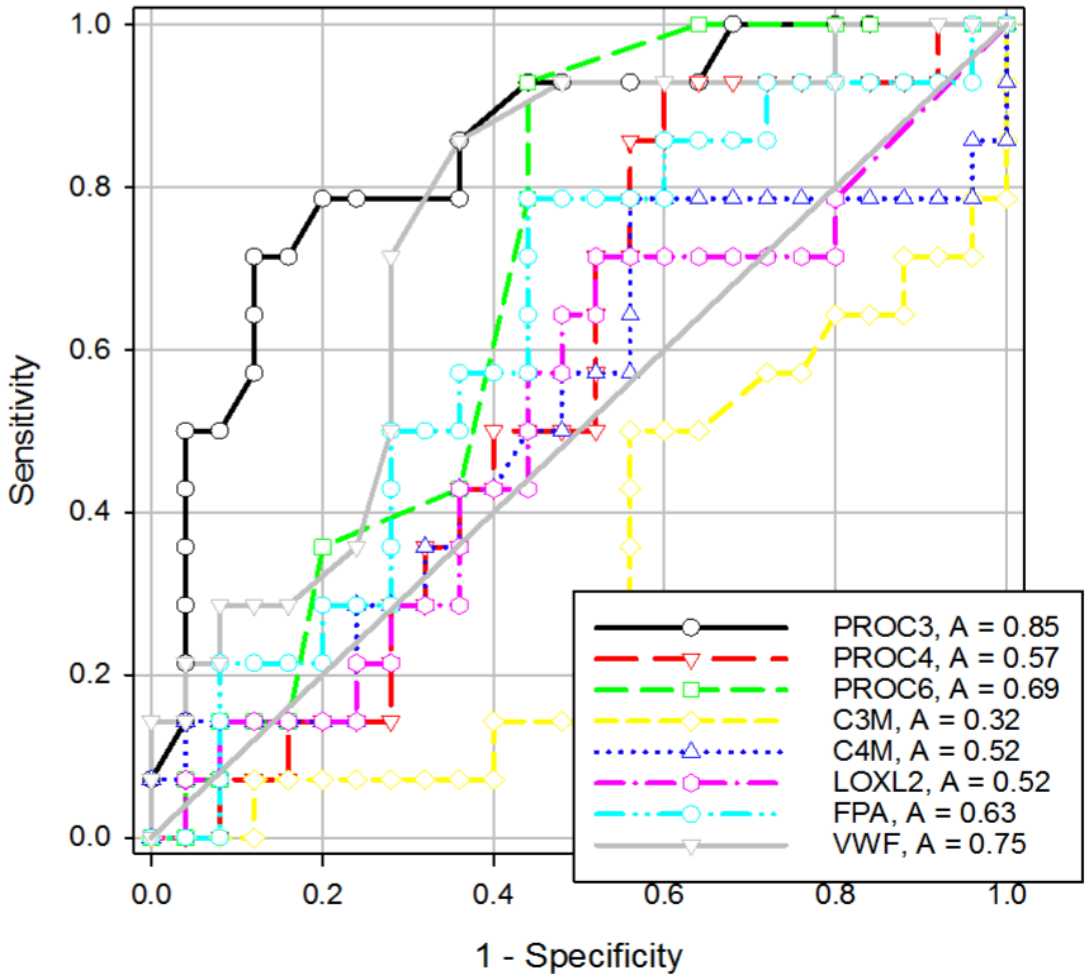


Figure 4.10 Biomarker AUROC for detection of advanced fibrosis, validation cohort (n=39)

4.4. DISCUSSION

Mortality in NAFLD is directly proportional to fibrosis stage, increasing exponentially when patients develop fibrosis stage ≥F3 (128) alongside NASH, an injurious process believed to accelerate disease progression. The current study assesses the diagnostic accuracy of circulating collagen fragments reflective of fibrogenesis and fibrolysis to determine fibrosis stage and the presence of NASH within the ***FDA Best diagnostic context of use***. It was found that elevated PROC3 and PROC6 levels were associated with advanced fibrosis and higher grades of steatohepatitis. The literature provides evidence that collagens (both fibre quantity and their spatial distribution) and their degradation products modulate fibrosis progression. Irrespective of understanding the intricacies of collagen metabolism at a molecular level, at a macroscopic level, clinical scientists can appreciate that collagen molecules, responsible for the increased ECM density observed in fibrosis harbour enormous potential for biomarker development (130).

4.4.1. Biological plausibility of the relationship between serum collagen fragment level and liver fibrosis

Progression to cirrhosis involves a tenfold increase in ECM collagens. In response to hepatic stellate cell activation (with a contribution from myofibroblasts and activated biliary and sinusoidal endothelia), the low density basement membrane (BM) like matrix occupying the Space of Disse mutates 'capillarization of the sinusoids' (481) accompanied by a non-uniform increase in all collagen sub-types (482). The end result is a net increase in type I over type III collagen with a decrease in type IV collagens (195). It has been shown that non-invasively monitoring serum/plasma levels of specific procollagen fragments can be used to evaluate the balance of fibrogenesis and fibrolysis (483). These procollagens and degradation products were investigated in this study (PROC3, PROC4, PROC6, C3M and C4M).

4.4.2. The future- Protein Fingerprint technology

Nordic bioscience have developed a PROC3 assay using an antibody engineered to recognise the neo-epitope at the cleavage site thus measuring collagen formation only i.e. active fibrogenesis unlike its predecessor PIIINP (456). In defining the context of use for a

biomarker it is important to appreciate that different sub-pools of collagen end-products provide different types of information. For example, in relation to type III collagen, PROC3 and C3M are representative of collagen formation and degradation respectively emphasising that vigilance is required to select the correct assay to evaluate a specific process. This was demonstrated in a study be Nielson et al, where a head to head comparison of PROC3 and PIIINP did not correlate showing the significance of biomarker specificity (456).

4.4.3. PROC3 and PROC6 as diagnostic biomarkers in active liver disease

In this study, moderate correlation was observed between the collagen formation biomarkers PROC3 and PROC6 (Rs=0.612). These fragments also displayed the best predictive power for identifying severe fibrosis (F3-F4) and features of steatohepatitis. The literature supports this trend (484). Research by Luo et al found that NAFLD patient serum levels of PROC3 were significantly higher (47%) in advanced disease (F3-F4) compared to mild disease (F0-F2) while serum levels of PROC6 were elevated by a level of 29% in comparison. In this study, we looked at PROC3 and PROC6 at a single context of use (Diagnostic). However, its efficacy in different contexts of use has been explored by other researchers in various liver diseases. ***Prognostic capacity*** in chronic Hepatitis C, patients with higher baseline PROC3 levels (>20.2ng/ml) showed progression of fibrosis while those with lower levels did not progress (449, 485). PROC3 (also C4M) levels have also been correlated with hepatic venous pressure gradient which is an invasive diagnostic and prognostic marker in cirrhosis and portal hypertension (476). ***Disease monitoring*** Longitudinal data in a cohort of fibrosis regressors has shown that in patients with higher baseline PROC3 levels (>16ng/ml), a steady decline in PROC3 levels with fibrosis regression was observed reflective of decreasing tissue injury. PROC3 has been shown to predict progression of liver disease and to identify responders to anti-fibrotic treatment in various aetiologies of liver disease in addition to NAFLD including HCV, HBV and alcoholic cirrhosis (166, 476, 485, 486). ***Diagnostic capacity*** In this study the diagnostic accuracies of PROC3 and PROC6 for the detection of advanced fibrosis included AUROC values of 0.78 and 0.76 respectively, with the ability of PROC3 and PROC6 to discriminate specific fibrosis stages improving in a stepwise manner with advancing disease. Negligible improvement was

observed when both biomarkers were combined (0.79). Only PROC3 maintained a statistically significant AUROC (0.63) for NASH detection and its levels also exhibited the strongest significant correlation (Rs= 0.388) with the CRN NAFLD Activity Score (NAS) Score. This study has demonstrated that PROC3 thus has the potential to fulfil the diagnostic context of use outlined in the FDA Best criteria for biomarker development with perhaps promise for use in a prognostic and dynamic capacity also. ***Improving performance*** in comparison to validated fibrosis indices, single biomarkers PROC3 (AUROC 0.76) and PROC6 (AUROC 0.73) performed equivalently to FIB 4 (0.78) and the NFS (0.80). To date, isolated parameters usually do not reach diagnostic accuracy indices that are significant and applicable in different cohorts (487). The modest performance of PROC3 in discriminating advanced fibrosis suggest that PROC3 as a single biomarker is sub-optimal for diagnosing static fibrosis stages, however a combination of PROC3 with other biomarkers may enhance its performance. A further consideration is that PROC3 may be more useful in identifying patients with active fibrogenesis than diagnosing static advanced disease stages as frequently patients with extensive amounts of scar tissue may have low levels of PROC3.

4.4.4. Inferior biomarker performance

PROC6 at a molecular level is a C-terminal pro-peptide of collagen type VI with high levels of expression in adipose tissue (460).This biomarker was in theory promising for the detection of advanced NASH as endotrophin predicts the response of HbA1c serum levels to insulin sensitizers (PPAR-gamma agonists) and predicts progression of kidney fibrosis (192, 488). However, this study failed to show that endotrophin was superior to PROC3 for NASH and fibrosis detection. A correlation of PROC6 and PROC3 and a significant trend of higher PROC6 levels in advanced fibrosis was observed in this study, however it is possible that the association of PROC6 with liver fibrosis is circumstantial and more correctly correlated with adipose fibrosis than liver damage (489).

The basement remodelling markers ***PROC4*** and ***C4M*** performed poorly. PROC4 did not reach significance for fibrosis or NASH detection and C4M, although significant for the detection of advanced fibrosis performed poorly (AUROC 0.59) compared to PROC6 and PROC3. One of the hallmarks of very advanced fibrosis/cirrhosis, reflected by PROC4 and

C4M levels is the development of a BM like structure on the Space of Disse which does not occur in the earlier fibrosis stages. The study cohort consisted of 38% patients with advanced disease therefore PROC4 and C4M fragments are likely to only be predictive in this small subset in contrast to PROC3 levels which are related to matrix remodelling occurring in all stages of fibrosis development (490).

C3M performed poorly as a biomarker. This was also observed in a study by Nielsen et al in cirrhotic HCV patients where C3M was unable to predict fibrosis progression (485). This repeat finding stresses the importance of clearly defining biomarker context of use as different sub-pools of the same collagen yields specific tissue turnover information.

In this study, degraded collagen fragments performed poorly as single biomarkers; however, their serum levels did increase incrementally with advancing disease stage. Degraded collagen fragments have been reported to catalyse an acute inflammatory response through neutrophil recruitment (491). With disease progression, cellular disposal of collagen fragments becomes less efficient resulting in an enhanced inflammatory response alongside the increased ECM accumulation. The increased levels of degradation fragments observed in later disease stages may therefore serve as a marker of disease activity (492).

Collagen binding, activation of platelets, fibrin clot formation and dissolution provide a framework for the tissue remodelling typical of fibrosis (493, 494). However, ***VWF*** and ***FPA*** associated with the collagen platelet axis in tissue remodelling did not show promise as biomarkers. There is literature to suggest that pro-thrombotic factor production may be driven by chronic inflammation associated with the metabolic syndrome (MetS) rather than by NASH associated necro-inflammation (495) ***LOXL2*** is responsible for collagen crosslinking and stability. Despite evidence that it is upregulated in fibrosis and tightly correlated with fibrosis stage (466, 496), this study failed to show significant correlation with advanced fibrosis or steatohepatitis. The non-collagen based biomarkers investigated in this study did not show any predictive power in this study cohort. A possible explanation might be that LOXL2 levels do not become discriminatory until cirrhosis with portal hypertension is well established which represented only a small subset of this study population (13/109= 12%).

4.4.5. Development of a simple blood test to stage NASH

Phlebotomy is simple and low risk in contrast to invasive liver biopsy and expensive imaging techniques. Blood tests developed with a specific context of use (fibrosis staging) may be useful. Collagen fragments levels, measured with neo-epitope specific antibodies have the potential to reflect a site-specific event unlike other molecules associated with a liver specific injury pattern that can come from alternative sources. For example, liver secreted CRP does not correlate with NASH because its production is stimulated by MetS associated chronic inflammation rather than NASH associated necro-inflammation (497). At best, the optimal serum based biomarker can stratify patients mild versus severe fibrosis. However, a simple blood test will never be able to parallel the complex information obtained from liver biopsy.

4.4.6. Strengths & Limitations

Sample collection This is a retrospective analysis involving samples collected over a long period of time (up to 10 years). The serum samples were collected in tertiary referral centre where careful attention was afforded to sample acquisition. These samples were stored at -80^{o}C which is thought to ensure stability. The samples were processed in a single centre where assay parameters were optimised to ensure the technical quality of each biomarker; minimising inter-assay variation and preventing false interpretation of results. Lack of sufficient serum in this study cohort meant that it was not possible to benchmark the collagen fragments with tests such as ELF, specifically looking at PIINP in line with PROC3. In a paper employing the ADVIA Centaur platform to assess PIIINP, a 2 fold difference in the measured levels were reported when a radioimmunoassay (UNiQ) was used compared with the new ADVIA Centaur immunoassay which has been tested as a component in the ELF test by Siemens health care diagnostic (452). Even if additional serum was available in this cohort, the lack of a standardised PIIINP assay and the fact that the PIINP antibodies used are often not disclosed (therefore may not be specific to the pro-peptide cleavage site) would present another potential problem.

Study cohort Both the discovery and validation cohort consisted of a broad spectrum of disease severity and complete information on metabolic parameters (T2DM, lipid profiles etc.) was available for inclusion in the multivariate analysis. However, there is the possibility

of referral bias given the patients were recruited from tertiary centres with an interest in NAFLD management.

Statistical analysis ROC Curves were generated as a measure of biomarker accuracy. However, liver biopsy is an imperfect reference standard, misclassifying disease stage with a frequency of 20% and furthermore, ROC curves provide no information on biomarker predictive values in clinical populations (114).

Biomarker Collagen fragments measure active collagen formation; however, they are not representative of the net fibrosis area in the liver. This may be one of the factors responsible for the lower performance of PROC3 in identifying fibrosis measured by histology than for example elastography quantified by MRE.

4.4.7. Future directions

Development of a PROC3 diagnostic panel PROC3 levels are significantly predictive of fibrosis stage, lobular inflammation and hepatocellular ballooning providing an evidence base to develop a diagnostic panel involving PROC3 (as a biomarker of disease activity/fibrogenesis) in combination with other well-established biological and metabolic parameters predictive of fibrotic disease. The recently Innovative Medicine Initiative (IMI)-granted Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS) consortium, which is associated with this study group, is an ideal platform to co-ordinate this research in an academic-industry collaborative effort. To further support precision medicine development, the FDA have developed the 'Accelerated Approval Pathway' that encourages biomarker development based on the opportunity to apply for drug approval with shorter studies based on validated biologically plausible biomarkers (498).

NAFLD progression signatures NAFLD exhibits vast inter-patient variability in disease progression. A non-invasive technology to stratify the NAFLD population into progressors/regressors will be valuable. Physiological characteristics and non-invasive tools in paired liver biopsy studies to date have not been predictive of those subjects likely to develop progressive disease. The ultimate biomarker package will likely be a combination of technologies addressing tissue formation and degradation in combination with an assessment of current tissue amount (likely provided by imaging) (499). This has been

achieved in osteoarthritis where serological markers and MRI outputs generate an Odds ratio of >20 for the likelihood of disease progression (500, 501).

Precision biomarker development Patterns of fibrosis are morphologically different in sub-types of liver disease. For example, the pattern in biliary fibrosis is distinct from that seen in chronic viral hepatitis which is again distinct from the 'chicken wire' pattern seen in ALD and NALFD (502, 503). If future basic science succeeds to elucidate the specific fibrogenic mechanisms and cell subtypes that drive the formation of distinct fibrosis patterns, the possibility for precision biomarker development exists.

4.5. CONCLUSION

The current study has established the utility of specific collagen fragments as biomarkers in the clinical care of NAFLD patients. PROC3 demonstrates the greatest diagnostic potential to identify patients with active fibrogenesis and may aid clinical trial recruitment.

CHAPTER 5.

PERFORMANCE OF THE PROC3 COLLAGEN NEO-EPITOPE BIOMARKER IN NON-ALCOHOLIC FATTY LIVER DISEASE

5.1. INTRODUCTION

5.1.1. NAFLD Pathogenesis

The Non-alcoholic fatty liver disease (NAFLD) spectrum includes simple steatosis affecting >5% of hepatocytes (NAFL) and non-alcoholic steatohepatitis (NASH), characterised by the presence of steatosis, lobular or portal inflammation, hepatocyte ballooning and fibrosis (52). Hepatocyte inflammation/ballooning and death are markers of disease activity (steatohepatitis), which drive the disease toward cirrhosis and are quantified by NAS score (45). Fibrosis is quantified using the 5 point scale developed by Brunt et al (48) which provides a numerical value (0-5) to indicate how far the disease has progressed on the pathway to cirrhosis. Satisfying the Food and Drug Agency (FDA) definition of "clinically meaningful benefit" in NAFLD patients at increased risk of disease progression involves reducing disease activity in the short term, and preventing progression to cirrhosis and thus adverse liver outcomes in the long-term. Progression to cirrhosis has been accepted by the FDA and European Medicine Agency (EMA) as part of a composite primary endpoint in current NASH marketing authorisation trials (137, 504). NASH resolution and improvement in fibrosis stage have both been accepted as surrogate endpoints "reasonably likely to predict clinical benefit" and have led to an accelerated/conditional drug approval pathways (505).

5.1.2. Histological determinants of NAFLD progression

Liver histology in NAFLD provides the basis for disease definition and both steatohepatitis (SH) and fibrosis stage have been linked to clinical outcomes. Fibrosis is the only histological feature that has been found to be independently associated with long-term prognosis. Advanced fibrosis stage confers an increased risk of progression to cirrhosis, liver failure and HCC and is associated with an increased likelihood of all-cause mortality (53, 54, 56, 128). Fibrosis regression has thus become an important target in most NASH related clinical trials alongside resolution of SH (129).

5.1.3. Currently available biomarkers

Blood-based non-invasive tests for fibrosis can be dichotomized into "indirect markers", including simple non-invasive fibrosis scores derived from clinical and biochemical indices such as the FIB4 Score and the NAFLD Fibrosis Score (NFS) (164, 165, 174, 506, 507), and

"direct biomarkers" that measure collagen deposition or matrix turnover (177, 508). The majority of non-invasive tests exhibit high negative predictive value, implying that they are best employed to exclude patients without advanced fibrosis (Kleiner ≤F2). However, many issues exist with currently available biomarkers. As described in chapter 1, FIB4 and NFS provide "indeterminate" results in a quarter of patients (115) and although elastography based techniques such as Fibroscan™ (vibration controlled transient elastography, VCTE) have a competitive diagnostic accuracy, they require specialist equipment, are operator dependent and exhibit low success rates in obese patients (509). Magnetic Resonance Elastography (MRE) can accurately diagnose fibrosis in NAFLD patients, but is expensive and not widely available (229, 510).

5.1.4. Diagnostic capacity of collagen neo-epitope biomarker PROC3

Collagens constitute the majority of pathological proteins accumulating in fibrosis in the ECM and represent promising candidates for the derivation of non-invasive serological fibrosis biomarkers (130, 483). Research exploiting knowledge of collagen structure and protease-protein interactions have resulted in the design of a specific enzyme-linked immunosorbent assay (ELISA) **Figure 6.1** (450, 456). PROC3 is an epitope of the NH_2 terminal pro-peptide of type III pro-collagen, that is released by protease mediated cleavage (ADAMS2-A Disintegrin and Metalloproteinase with Thrombospondin Motifs) during collagen maturation and subsequent incorporation into collagen type III in collagen fibrils (511). In chapter 5, a panel of collagen fragments were investigated as potential biomarkers and PROC3 performed best for both NASH (AUROC 0.69/0.63) and advanced fibrosis (AUROC 0.78/0.73) detection in the discovery and validation cohort respectively. This is supported by the literature where there is evidence that PROC3 can identify fast fibrosis progressors and treatment responders in multiple aetiologies of liver disease beyond and including NAFLD (166, 476, 485, 486, 512) Previous studies have shown that measuring formation of type III collagen neo-epitopes (PROC3) and incorporation into a diagnostic panel can provide a reasonably accurate assessment of disease stage and activity but to date require complex mathematical calculations (166, 479, 511, 513, 514). Similarly, NFS and FIB4 require the use of online calculators to generate a result. This may be onerous in a busy clinical environment and limits adoption in primary care settings (515, 516). A

simplified but accurate fibrosis assessment algorithm would therefore assist physicians to risk stratify patients without recourse to an online calculator.

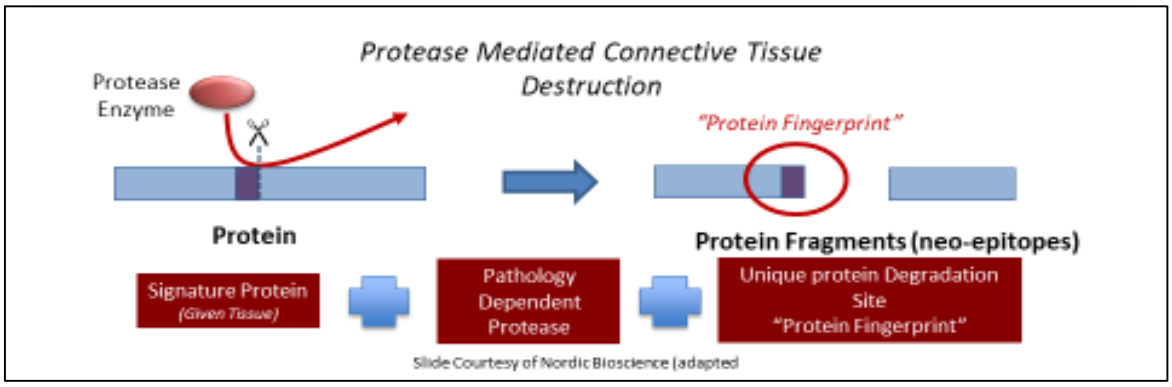


Figure 5.1 Protein Fingerprint Technology

5.1.5. Biomarkers- Diagnostic context of use

The BIPED (Burden of disease, Investigative, Prognostic, Efficacy of intervention, Diagnostic) criteria has been proposed by the US National Institutes of Health (NIH)-funded Osteoarthritis (OA) Biomarkers Network where the developed biomarker must be compared to one or more gold standards for disease assessment. However, liver biopsy similar to joint space width in OA, as a gold standard is imperfect making biomarker development challenging (517).

The FDA mandates that the context of use of a biomarker be clearly defined at the outset of development. Defining the context of use of a biomarker involves derivation of a (1) use statement and defining (2) the conditions for qualified use. The BEST glossary defines seven categories of biomarkers and each biomarker category can have multiple contexts of use. In this study, PROC3 has been provisionally defined as a diagnostic biomarker (stage of fibrosis) but has the possibility of also being defined as a prognostic biomarker (stratify individuals by fibrosis stage, to predict long-term outcomes and hard end points) and a dynamic 'monitoring' biomarker (to track progression of disease severity/ track response to therapy or efficacy of intervention). If the study hypothesis is proven, this can qualify PROC3 as a biomarker with a "limited" context of use and so facilitate its integration into drug development. This will allow for further accumulation of supportive data on its use and for it to progress to an "expanded" context of use.

5.1.6. Genetic biomarkers

Genetic susceptibility plays a prominent role in NAFLD pathogenesis and a number of variants have been identified that affect the severity and progression of NAFLD (201-205, 433-437) . A well characterised example is the G allele in the forward strand of s738409 C/G -a variant nonsynonymous single nucleotide polymorphism (SNP) of PNPLA3 (patatin-like phospholipase domain containing 3 (518, 519). Genetic markers have been interrogated as standalone disease severity biomarkers or as part of a diagnostic panel to predict hepatic steatosis (NAFLD liver fat score) (520), to distinguish NASH from NAFL (NASH Clin score and NASH ClinLipMet score) (521, 522) with modest discriminatory area under the receiver operating curves (>0.75). Panels involving genetic markers have also been investigated as tools to monitor disease response to interventions such as low carbohydrate hypocalorific diets, structured lifestyle modification programs and bariatric surgery (523-525). As a standalone biomarker, the PNPLA3 GG genotype was predictive of stage 2 fibrosis yet lost power beyond stage 3 perhaps suggesting that the impact of the *PNPLA3* variant tended to decrease in patients with severe fibrosis stage e.g. burnt out NASH (526). To date, PNPLA3 genotype has not been incorporated into a diagnostic panel for fibrosis detection. This will be investigated in a subsection of this study.

5.1.7. Study Objectives

In the current study, the objectives are to:

- Assess the performance of PROC3 as a NASH-fibrosis biomarker ***within the BEST diagnostic context of use***
- Develop and validate a novel biomarker panel incorporating PROC3 and determine its performance in comparison to established clinical scores and previously reported biomarker panels
- Develop and validate a simplified clinical tool that is both accurate and clinically accessible immediately
- Investigate if the addition of PNPLA3 genotype improves the diagnostic accuracy of a PROC3 based fibrosis detection model.

5.2. METHODS

5.2.1. Study Design and Participants

Figure 6.2 shows the flow of patients through the study. Subjects were recruited at seven specialist European centres as described in chapter 3.

5.2.2. Clinical and laboratory assessments

Clinical and laboratory assessments were performed as described in chapter 3. Calculation of fibrosis indices as described in chapter 5.

5.2.3. Histological Assessment

Histological assessment was performed as described in section 2.2. To reduce the element of inter-observer variability, over half of all biopsies (254, 57%) in our study were centrally reviewed by an expert member of the EPoS Histopathology Group (DT). A weighted kappa co-efficient of 0.90 for fibrosis stage was established, demonstrating a very high level of inter-observer agreement.

5.2.4. Statistical Analysis

The primary endpoint of the study was to predict the presence of advanced fibrosis (stages 3-4). The combined cohort of 449 patients was randomly separated into approximately 1/3 (n=151) (Discovery cohort) and 2/3 (n=298) of patients (Validation cohort) for model building and validation (FIBC3 and ABC3D). A cohort of 358 patients was randomly separated into approximately 2/3 (n=234) and 1/3 (n=124) validation cohort for model building and validation of the PROC3PNPLA3 model. Continuous variables were compared using the t-test and categorical variables using Chi-square test. The Kruskal-Wallis test was used to perform comparisons between mean marker levels followed by Dunn's multiple comparison tests. In the discovery cohort, significant variables on univariate analysis ($p<0.05$) were included in the backward stepwise multiple logistic regression analysis to identify independent factors associated with fibrosis. Variables with $P < 0.05$ by multivariate analysis were used to construct scoring systems (FIBC3, ABC3D and PROC3PNPLA3) to predict advanced fibrosis. Optimal cut-offs for each component of ABC3D were selected using the Youden index (J-Index) which attributes equal value to sensitivity and specify. Cross-validation was performed

using the leave one out method to facilitate the calculation of over-fit bias reduced estimates. We calculated reduced bias estimates of predicted probability. This involved removing each individual subject and re-estimating the model parameters and then classifying the subject based on the new parameters. This enabled us to interrogate a suspicious positive or negative validation subject.

The diagnostic accuracies of both scoring systems were determined by calculating the area under the receiver operating characteristic (ROC) curve (the c-statistic) and its 95% confidence intervals. The 5-point fibrosis scales presented both spectrum effect and ordinal scale issues. To overcome this, we calculated the Obuchowski measure using the package "nonbinROC" version 1.0.1 (https://CRAN.R-project.org/package=nonbinROC) using the R statistical analysis software platform (527). This is a measure of the probability that our fibrosis index will correctly rank 2 randomly chosen patient samples from different fibrosis stages according to the weighting scheme, with a penalty score of 1 for incorrect scoring (528). The method of DeLong, DeLong and Clarke-Pearson was used to compare AUROCs (529). Validation was performed in (1) the validation datasets and (2) in the full datasets. Using the ROC curve for the final model, a cut-off point was selected using the Youden Index (J-Index). ROC curves were also calculated for the established fibrosis indices, calculated as described in section 2.2 [10, 24, 27-29]. All statistical analyses were performed using SPSS software version 24.0 (SPSS Inc, Chicago, USA), R and SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).

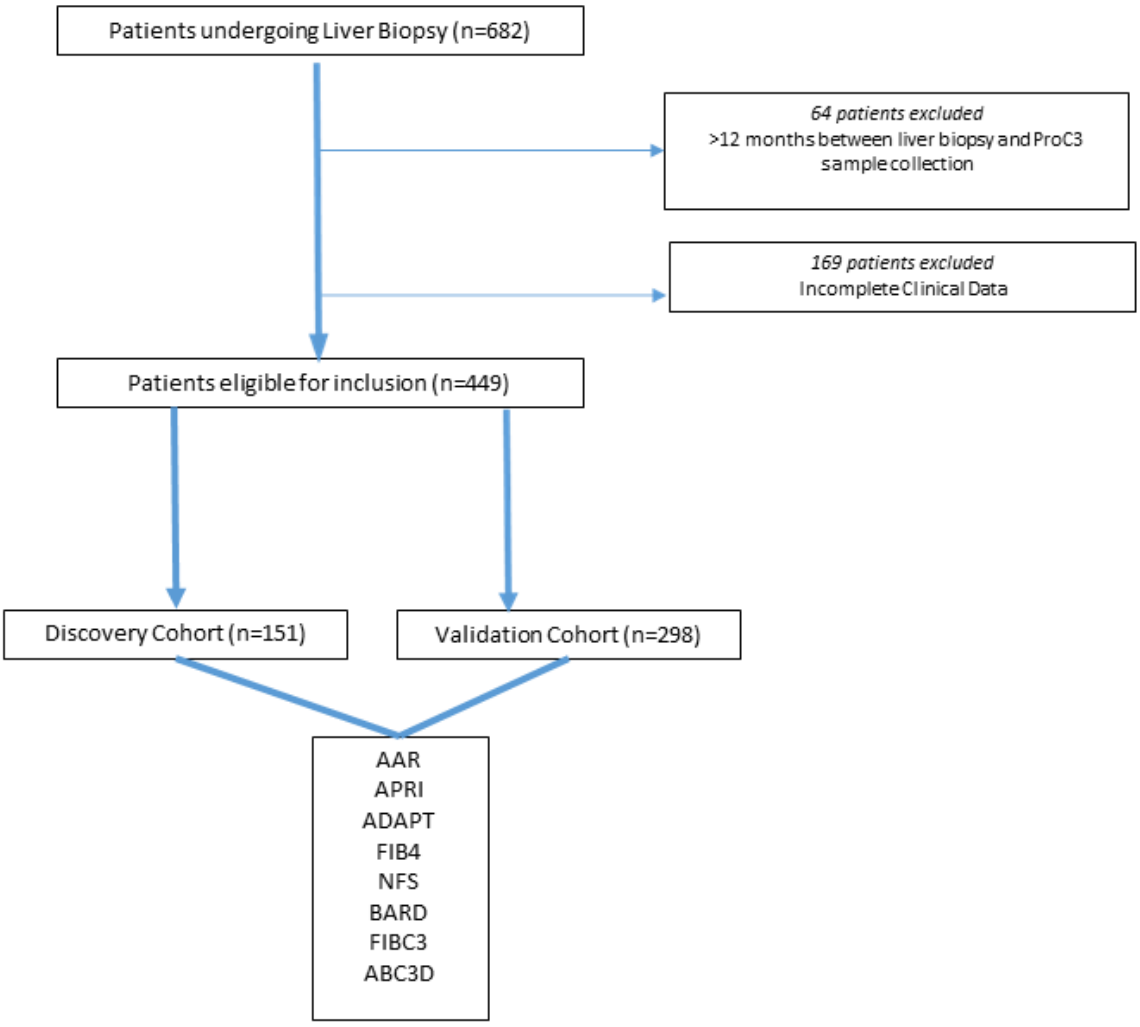


Figure 5.2. Patient flow

AAR= AST to ALT ratio; APRI= AST to platelet ratio index; ADAPT= Age, Diabetes, PRO-C3 and platelets panel; FIB 4 = Fibrosis 4 Index; NFS= NAFLD Fibrosis Score; BARD= BMI, AST/ALT Ratio, Diabetes; FIB C3= Fibrosis

5.3. RESULTS

5.3.1. Characteristics of Patient Population

Table 5.1 summarizes the clinico-demographic details of the study population. The 449 patients were pooled from six international centres **(Table 5.2)**. No country of origin/centre effect was detected in the analysis (p=1.000).

Table 5.1 **Baseline Demographic and Clinical characteristics of participants**				**Discovery vs. Validation Cohort**
Variable	**All Patients (n=449)**	**Discovery Cohort (n=151)**	**Validation Group (n=298)**	**P-Value**
Age (years)	52+/-13	51.6+/-13	51.5+/-13	*0.957*
Gender (male)	263 (59%)	94 (62%)	169 (57%)	*0.260*
BMI (Kg/m^2)	32.6+/-6.8	32.9+/-7.1	32.4+/-6.4	*0.608*
T2DM	216 (48%)	74 (49%)	142 (48%)	*0.786*
ALT (U/l)	69+/-41	66+/-39	71+/-42	*0.166*
High ALT (>40U/l)	340 (76%)	112 (74%)	228 (77%)	*0.585*
AST (U/l)	47+/-26	47+/-26	48+/-26	*0.339*
Albumin (g/dl)	44+/-5	44+/-4	44+/-5	*0.780*
Platelets (X10^9/l)	230+/-72	225+/-61	233+/-77	*0.448*
Cholesterol (mg/dl)	7+/-14	7+/-10	7.1+/-16	*0.630*
Triglycerides (mg/dl)	3.8+/-17	3.6+/-16	3.9+/-18	*0.758*
Collagen PROC3(ng/ml)	18.9+/-15	18.1+/-14	19.3+/-15	*0.438*
Collagen PROC6(ng/ml)	9.6+/-4.4	9.3+/-4	9.8+/-4.7	*0.501*
PROC4 (ng/ml)	266+/-142	253+/-147	273+/-139	*0.067*
C4M (ng/ml)	27.3+/-10	26.8+/-10.1	27.6+/-9.8	*0.374*
C3M (ng/ml)	11.6+/-4	11.6+/-4.8	11.6+/-4.2	*0.644*
Fibrosis Stage (0/1/2/3/4)	90/100/92/101/66	36/28/27/34/26	54/72/65/67/40	*0.309*
Steatosis 0/1/2/3	10/149/171/110	6/50/56/35	4/99/115/75	*0.342*
Ballooning 0/1/2	112/188/138	38/60/49	74/128/89	*0.791*
Lobular Inflammation 0/1/2/3	48/219/147/24	18/78/43/8	30/141/104/16	*0.578*
NAS	4+/-2	4+/-2	4+/-2	*0.848*
FIB4	1.53+/-1.07	1.55+/-1.08	1.52+/-1.06	*0.483*

AAR	0.76+/-0.31	0.79+/-0.34	0.75+/-0.30	*0.428*
NAFLD Fibrosis Score	-1.304+/-1.796	-1.182+/-1.797	-1.367+/-1.795	*0.303*
APRI	0.68+/-0.48	0.68+/-0.51	0.68+/-0.46	*0.718*
ADAPT Score	6.3+/-2.2	6.3+/-2.3	6.4+/-2.2	*0.652*
BARD Score	2+/-1	2+/-1	2+/-1	*0.428*
Centrally Reviewed Biopsies	254 (57%)	79 (52%)	175 (59%)	0.622

The table shows the mean ± SD for continuous variables, number (%) for binary variables, and number per group for categorical variables NAS= NAFLD Activity Score
Statistical test; T-Test/Mann Whitney was used to test for significant differences within continuous variables and Chi-Square test was used for categorical variables.
BMI= Body mass index; T2DM= Type 2 Diabetes Mellitus; ALT = Alanine Aminotransferase; AST= Aspartate aminotransferase; NAS = NAFLD activity score; FIB4= Fibrosis-4 Index; AAR= AST to ALT ratio; APRI= AST to platelet ratio index. ADAPT = Algorithm including Age, Diabetes, PROC3 and platelet count. BARD = BMI, AST/ALT ratio, Diabetes

Table 5.2. Study site contribution to total cohort

Total Cohort (n=449)			**Discovery Cohort (n=151)**		**Validation Cohort (n=298)**	
Study Site	N	%	N	%	N	%
USP	14	3	2	1	12	4
UP	90	21	30	20	60	21
UNITO	95	21	34	23	61	20
UNEW	160	35	49	32	111	37
UM	54	12	17	11	37	12
ICAN	36	8	19	13	17	6

*USP= University of Sao Paulo School of Medicine; UP= University of Palermo; UNITO= University of Torino; UNEW= Newcastle University; UM= University Hospital Mainz; ICAN =Institute of Cardiometabolism and Nutrition Paris: **P-value = 1.000 Discovery versus Validation cohort**

5.3.2. PROC3 levels correlated with steatohepatitis and fibrosis stage

Across all histological features (steatosis, lobular inflammation, hepatocyte ballooning, fibrosis), PROC3 was positively associated with increasing NAFLD severity **(Figure 5.3).** In the discovery cohort (n=151), PROC3 correlated with NAS score (Rs=0.304, p<0.0001) and fibrosis stage (Rs=0.422, p<0.0001). Confirming that PROC3 is primarily a fibrosis marker, the correlation with fibrosis stage remained significant when controlling for NAS however the converse did not hold true. Indeed, PROC3 exhibited the strongest correlation with fibrosis

stage when compared to a number of other putative ECM turnover biomarkers (PROC6 (Rs=0.355), PROC4 (Rs= 0.279) and C4M (Rs=0.177), p<0.05).

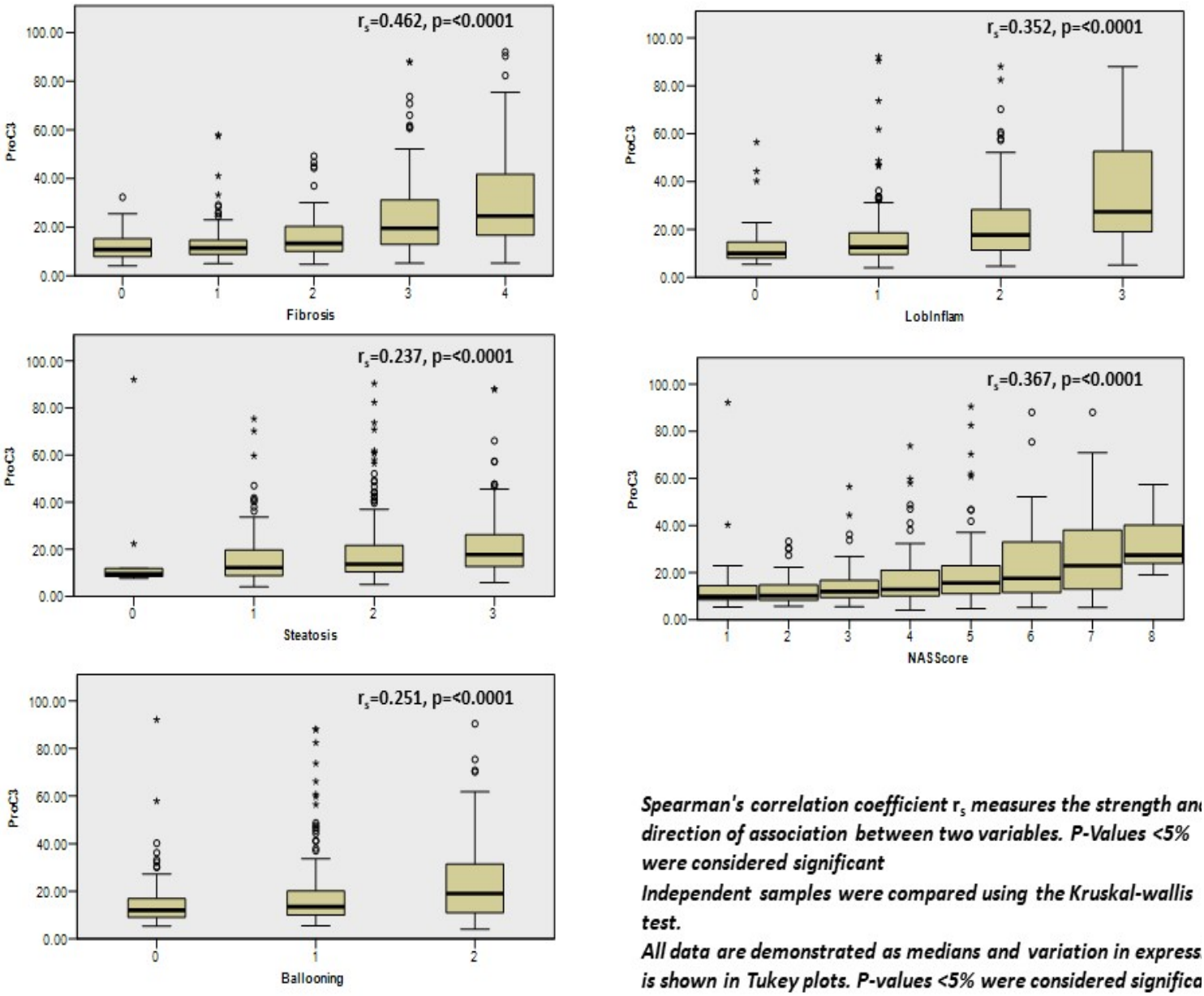


Figure 5.3. PROC3 and its association with NAFLD Severity (Complete Cohort n=449)

In the discovery cohort (n=151) an optimal PROC3 cut-off level for the detection of advanced fibrosis was determined. PROC3 >15.5ng/ml had an AUROC of 0.73 for the detection of advanced fibrosis ≥F3 (sensitivity 60%, specificity 74%, accuracy 68%). This was replicated in the validation cohort (n=298) (AUROC = 0.78, sensitivity 72%, specificity 71%, accuracy 71%) **(Table 5.3)**. The previous study had identified a cut-off >17.5ng/ml for the detection of

advanced fibrosis. The sensitivity and specificity for fibrosis across a range of PROC3 thresholds are reported for the overall cohort (**Table 5.4**).

Table 5.3: Exploration of PROC3 as a single biomarker

PROC3 for the Detection of NASH and Advanced Fibrosis

Optimal PROC3 cut off for detection of advanced fibrosis (F≥3): PROC3>15.5ng/ml
AUROC 0.73 (0.652-0.812)

	PPV	**NPV**	**Sensitivity**	**Specificity**	**Accuracy**
Discovery Cohort (n=151)	60	74	60	74	68
Validation Cohort (n=298)	58	82	72	71	71
FIBC3 Validation Cohort (n=298)	63	84	75	75	75

Optimal PROC3 cut off for detection of *tdNASH with ≥F2 fibrosis: PROC3>14.5ng/ml
AUROC 0.68 (0.591-0.761)

	PPV	**NPV**	**Sensitivity**	**Specificity**	**Accuracy**
Discovery Cohort (n=151)	63	65	59	69	64
Validation Cohort (n=298)	64	74	70	68	69
FIBC3 Validation Cohort (n=298)	63	84	75	75	75

Optimal PROC3 cut off for detection of *tdNASH with F4 cirrhosis: PROC3>16.5ng/ml
AUROC 0.68 (0.535-0.817)

	PPV	**NPV**	**Sensitivity**	**Specificity**	**Accuracy**
Discovery Cohort (n=151)	24	95	74	67	68
Validation Cohort (n=298)	20	95	76	61	63
FIBC3 Validation Cohort (n=298)	63	84	75	75	75

*tdNASH = “trial definition NASH” = steatosis with a NAS≥4 with at least 1 point each for steatosis, hepatocyte ballooning and hepatic inflammation and stage ≥F2 fibrosis (or F4 for tdNASH-Cirrhosis)

Table 5.4: Performance of PROC3 as a Diagnostic Biomarker at Different Threshold Levels												
	TP	**TN**	**FP**	**FN**		**PPV**	**NPV**	**SEN**	**SPEC**	**ACCURACY**	**Actual No.Cases**	**TP(%)**
PROC3>5												
tdNASHF1234	282	0	163	4	449	63	0	99	0	63	286	99
tdNASHF0	15	3	430	1	449	3	75	94	1	4	16	94
tdNASHF1	59	2	386	2	449	13	50	97	1	14	61	97
tdNASHF2	68	3	377	1	449	15	75	99	1	16	69	99
tdNASHF3	87	4	358	0	449	20	100	100	1	20	87	100
tdNASHF4	53	4	392	0	449	12	100	100	1	13	53	100
Advanced Fibrosis	167	4	278	0	449	38	100	100	1	38	167	100
PROC3>10												
tdNASHF1234	235	65	98	51	449	71	56	82	40	67	286	82
tdNASHF0	11	111	322	5	449	3	96	69	26	27	16	69
tdNASHF1	38	93	295	23	449	11	80	62	24	29	61	62
tdNASHF2	56	103	277	13	449	17	89	81	27	35	69	81
tdNASHF3	82	111	251	5	449	25	96	94	31	43	87	94
tdNASHF4	48	111	285	5	449	14	96	91	28	35	53	91
Advanced Fibrosis	151	100	182	16	449	45	86	90	35	56	167	90
PROC3>15												
tdNASHF1234	152	115	48	134	449	76	46	53	71	59	286	53
tdNASHF0	4	237	196	12	449	2	95	25	55	54	16	25
tdNASHF1	15	203	185	46	449	8	82	25	52	49	61	25
tdNASHF2	36	216	164	33	449	18	87	52	57	56	69	52
tdNASHF3	57	219	143	30	449	29	88	66	60	61	87	66
tdNASHF4	40	236	160	13	449	20	95	75	60	61	53	75
Advanced Fibrosis	115	197	85	52	449	58	79	69	70	69	167	69
PROC3>20												
tdNASHF1234	106	140	23	180	449	82	44	37	86	55	286	37
tdNASHF0	1	305	128	15	449	1	95	6	70	68	16	6
tdNASHF1	9	268	120	52	449	7	84	15	69	62	61	15
tdNASHF2	21	272	108	48	449	16	85	30	72	65	69	30
tdNASHF3	44	277	85	43	449	34	87	51	77	71	87	51
tdNASHF4	31	298	98	22	449	24	93	58	75	73	53	58
Advanced Fibrosis	88	241	41	79	449	68	75	53	85	73	167	53
PROC3>25												
tdNASHF1234	15	208	148	78	449	9	73	16	58	50	286	5
tdNASHF0	1	341	92	15	449	1	96	6	79	76	16	6
tdNASHF1	5	300	88	56	449	5	84	8	77	68	61	8
tdNASHF2	11	298	82	58	449	12	84	16	78	69	69	16
tdNASHF3	35	304	58	52	449	38	85	40	84	76	87	40
tdNASHF4	26	329	67	27	449	28	92	49	83	79	53	49
Advanced Fibrosis	71	260	22	96	449	76	73	43	92	74	167	43

*tdNASH= NAS ≥4 with at least 1 point each for steatosis, hepatocyte ballooning and hepatic inflammation
**TP(%)= true positives/actual number of cases

5.3.3. Development of panels incorporating PROC3 that are diagnostic for advanced fibrosis

To identify other clinical factors that readily predict presence of fibrosis, additional analyses were conducted. **Table 5.5** shows the results of univariate and multivariate analyses preformed in the discovery cohort. Using backward logistic regression, five variables remained significantly associated with advanced fibrosis: age, BMI, T2DM, platelets and PROC3. No multi-collinearity was identified between variables used in the model. Variables were assessed for all two-way interactions with no significant outcomes ($p>0.05$). These five variables were incorporated into a model that distinguished advanced fibrosis (F3-4) from mild fibrosis (F0-F2). The diagnostic panel "FIBC3" was calculated from the regression formula for prediction of severity of fibrosis: -5.939 + (0.053*Age) + (0.076*BMI) + (1.614*T2DM) – (0.009*platelets) + (0.071*PROC3). FIBC3 correlated strongly with fibrosis stage ($Rs= 0.630$, $p<0.0001$), which remained significant independent of NAS. In the Discovery cohort, the area under the receiver operating characteristic curve (AUROC) for FIBC3 was 0.89 (95%CI 0.843-0.941, $p<0.001$).

Table 5.5 Variables Associated with the Presence of Advanced Fibrosis (stage F3-4) in the Discovery Cohort (n=151)

	Univariate			Adjusted (Multivariate)		
Variable	**Odds Ratio**	**95% CI**	**p-value**	**Odds Ratio**	**95% CI**	**p-value**
Age	1.088	1.049-1.128	<0.0001	1.055	1.008-1.103	0.022
Gender	1.172	0.599-2.291	0.643			
BMI	1.090	1.035-1.148	0.001	1.079	1.014-1.148	0.017
T2DM	8.570	4.003-18.348	<0.0001	5.023	1.920-13.140	0.001
ALT	1.002	0.994-1.011	0.611			
AST	1.020	1.005-1.034	0.007			
Albumin	0.934	0.853-1.021	0.133			
Platelets	0.986	0.986-0.979	<0.0001	0.991	0.982-1.000	0.039
Cholesterol	0.841	0.714-0.990	0.038			
Triglycerides	1.024	0.952-1.101	0.520			
PROC3	1.079	1.039-1.120	<0.0001	1.074	1.023-1.127	0.004
AST-ALT Ratio	3.072	1.119-8.436	0.029			

FIBC3:
-5.939+ (0.053*Age) + (0.076*BMI) + (1.614*T2DM) - (0.009*platelets) + (0.071*PROC3)
ABC3D:
Age>50 = 1 point, **B**MI>30 = 1 point, platelet **C**ount<200 = 1 point**,** PROC3>15.5=1 point, **D**iabetes = 2 points

To facilitate adoption in a clinical setting, a simplified score based on the same 5 variables identified as significant on univariate analysis and weighted according to their odds ratio (OR) values was generated. The derived "ABC3D" score comprises: **A** = **A**ge>50 years, **B** = **B**MI>30, **C** = platelet **C**ount<200, **3** = PROC**3**>15.5ng/ml, **D**iabetes = present. Optimal thresholds for each variable were selected by maximising the Youden index for the corresponding ROC curves. The presence of each factor scored 1 point, except for T2DM which, with an OR of 5, was awarded 2 points to yield a maximum score of 6. In the discovery cohort, the AUROC for ABC3D was 0.88 (95%CI 0.822-0.929, p<0.001).

5.3.4. Validation of FIBC3 and ABC3D model accuracy and derivation of diagnostic thresholds for advanced fibrosis

The diagnostic accuracy of these models for the detection of advanced fibrosis was confirmed in a validation cohort (n=298) and also in the overall combined cohort (n=449). Diagnostic accuracy was assessed by the standard AUROC and also the weighted AUROC computed using the Obuchowski measure to account for spectrum effect and ordinal scale. For FIBC3, the AUROC remained high in both the validation cohort (0.83, 95%CI 0.777-0.880) and the combined cohort (0.85, 95%CI 0.812-0.886). The weighted AUROC was calculated to be 0.77, 0.75 and 0.79 in the combined, discovery and validation cohorts respectively. Similar results were obtained for ABC3D with AUROC of 0.81 and 0.83 in the validation and combined cohorts respectively **(Table 5.6)**. Reduced bias estimates of predicted probability were calculated in the discovery and validation cohorts, employing the leave-one-out method of cross-validation as previously described. To assess the true value of PROC3, we removed PROC3 from the FIBC3 diagnostic model. This yielded AUROCs of (0.80, 0.86 and 0.76) in the total, discovery and validation cohorts respectively which improved to (0.85,0.89 and 0.83) with the inclusion of PROC3 in the model.

Table 5.6 Diagnostic Accuracy of Non-invasive Tests for Detecting Histologic Stage F3–F4 and Weighted AUROC Derived from the Obuchowski Measure															
Combined Cohort (n=449)						**Discovery Cohort (n=151)**					**Validation Cohort (n=298)**				
Non-invasive Test	**AUROC**	**95% CI**	**Adj AUROC**	**SD**	**95% CI**	**AUROC**	**95% CI**	**Adj AUROC**	**SD**	**95% CI**	**AUROC**	**95% CI**	**Adj AUROC**	**SD**	**95% CI**
AAR	0.67	0.615-0.716	0.62	0.019	0.581-0.653	0.66	0.579-0.751	0.62	0.031	0.555-0.675	0.66	0.599 - 0.725	0.62	0.024	0.571-0.663
APRI	0.75	0.698-0.794	0.68	0.017	0.652-0.717	0.75	0.669-0.830	0.69	0.028	0.638-0.748	0.75	0.686 - 0.805	0.68	0.021	0.640-0.722
BARD	0.71	0.664-0.761	0.67	0.017	0.642-0.707	0.76	0.683-0.834	0.69	0.028	0.637-0.746	0.69	0.624 - 0.749	0.66	0.021	0.623-0.705
FIB4	0.78	0.732-0.820	0.70	0.015	0.671-0.731	0.80	0.726-0.867	0.70	0.026	0.651-0.751	0.76	0.707 - 0.819	0.70	0.019	0.644-0.739
NFS	0.79	0.751-0.838	0.72	0.015	0.694-0.752	0.85	0.791-0.911	0.71	0.023	0.669-0.758	0.76	0.701 - 0.818	0.73	0.019	0.692-0.766
ADAPT	0.85	0.815-0.888	0.77	0.014	0.739-0.794	0.86	0.800-0.917	0.74	0.025	0.695-0.793	0.85	0.803 - 0.896	0.78	0.017	0.749-0.815
PROC3	0.76	0.718-0.811	0.69	0.017	0.660-0.726	0.75	0.661-0.831	0.68	0.031	0.617-0.740	0.78	0.727 - 0.838	0.70	0.020	0.622-0.741
FIB-C3	0.85	0.812-0.886	0.77	0.013	0.745-0.797	0.89	0.843-0.941	0.75	0.021	0.707-0.789	0.83	0.777 - 0.880	0.79	0.017	0.753-0.819
ABC3D	0.83	0.793-0.868	0.76	0.013	0.730-0.783	0.88	0.822-0.929	0.75	0.022	0.704-0.790	0.81	0.76-0.856	0.76	0.017	0.730-0.795
P-Value	<0.0001					**P-Value**	<0.0001				**P-Value**	<0.0001			
***Prevalence advanced fibrosis *combined cohort = 0.37 *Discovery cohort = 0.40 * Validation cohort = 0.36** ***DeLong DeLong Clarke test for comparison of AUROC**															

An optimal FIBC3 threshold value of >-0.4 was chosen using the Youden index (sensitivity 83%, specificity 80%, PPV 74% and NPV 88%). An optimal ABC3D cut-off level for the detection of advanced fibrosis was >3. In the validation cohort (n=298), FIBC3 exhibited a sensitivity 75%, specificity 75%, accuracy 75% **(Table 5.7)**. In the discovery cohort, ABC3D exhibited a sensitivity 77%, specificity 82%, and accuracy 80%. This was replicated in the validation cohort, where a sensitivity of 66%, specificity 75% and accuracy 73% was observed.

Table 5.7

Optimal Cut-off values for the detection of advanced fibrosis (≥F3) as per Youden Index (YI) derived in Discovery Cohort (Prevalence 0.40, n=151)
Application in Validation Cohort (Prevalence 0.36, n=298)

Panel	AUC	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
FIB-C3	0.89	>-0.4	83	80	74	88	81
ABC3D	0.88	>3	77	82	74	84	80
Validation Cohort							
	Cut-off	**Sensitivity (%)**		**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Accuracy (%)**
AAR	>0.8	46		71	47	70	62
APRI	>1.5	11		96	63	66	66
BARD	≥2	76		51	47	79	60
FIB4	>2.67	21		94	67	68	68
NFS	>0.676	27		95	78	70	71
ADAPT	>6.3	76		75	63	86	76
FIB-C3	>-0.4	75		75	62	84	75
ABC3D	>3	66		75	61	80	73

AAR= AST to ALT ratio; APRI= AST to platelet ratio index; BARD= BMI, AAR, T2DM; FIB 4 = Fibrosis 4 Index; NFS= NAFLD Fibrosis Score; ADAPT= Age, Diabetes, PROC3 and platelets panel; FIB C3= Fibrosis PROC3 Panel, ABC3D= Age, BMI, platelet Count, Diabetes, PROC3

Both FIBC3 and ABC3D performance was superior to simple non-invasive scores in current use with accuracies of 75% and 73% respectively. Performance characteristics of FIBC3 and the simplified ABC3D score were comparable to the recently described ADAPT score **(Table 5.7)**. Comparing AUROC curves using the DeLong, DeLong and Clarke-Pearson method confirmed that FIBC3 and ABC3D have similar performance characteristics (p = 0.1422) as do FIBC3 and ADAPT (p=0.1859). Using the FIBC3 model, the optimal threshold correctly staged 224 out of

298 patients (75%) in the validation cohort, compared to 227 patients (76%) with ADAPT and 217 (73%) with ABC3D. Considering NPV, of 191 patients with mild fibrosis, 144 (75%) were staged correctly using FIBC3 or ABC3D, equal to ADAPT (75%) (**Table 5.8**). In the combined cohort (n=449), 347 of the patients (77%) were correctly staged using FIBC3, which out-performed both FIB4 at 304 (68%) and ADAPT at 341 (76%). The most simple model, ABC3D, had a diagnostic accuracy of 75% correctly classifying 338 cases into mild or severe fibrosis.

Table 5.8 Validation cohort divided into mild and severe fibrosis (Prevalence 0.39, n=298)

	F0-2 'Rule out' advanced fibrosis				**F3-4 'Rule in' advanced in severe**		
	Correctly identified	Indeter minate	Incorrectly Identified		Correctly identified	Indeter minate	Incorrectly Identified
N=191				N=107			
AAR AAR<0.8	135/191 71%		56/191 29%	**AAR AAR>0.8**	49/107 46%		58/107 54%
APRI APRI<0.5	112/191 59%	72/191 38%	7/191 3%	**APRI APRI>1.5**	12/107 11%	72/107 67%	23/107 22%
BARD BARD<2	98/191 51%		93/191 49%	**BARD BARD>2**	81/107 76%		26/107 24%
FIB4 FIB4<1.3	133/191 70%	47/191 25%	8/188 5%	**FIB4 FIB4>2.67**	22/107 20%	53/107 50%	32/107 30%
NFS NFS<-1.433	120/191 64%	63/191 33%	5/191 3%	**NFS NFS>0.676**	29/107 27%	51/107 48%	27/107 25%
ADAPT ADAPT<6.3	144/191 75%		47/191 25%	**ADAPT ADAPT>6.3**	83/107 78%		24/107 22%
FIBC3<-0.4	144/191 75%		47/191 25%	**FIBC3>-0.4**	80/107 75%		27/107 25%
ABC3D <3	144/191 75%		47/191 25%	**ABC3D>3**	73/107 68%		34/107 32%

5.3.5. Performance of FIBC3 and ABC3D in real-world settings

The performance of FIBC3 and ABC3D was assessed in a range of pre-test probability scenarios that may be encountered across primary care and specialist care environments where the prevalence of advanced fibrosis varies to see if they were equivalent. The PPV and NPV were calculated across an advanced and mild fibrosis prevalence range between 5-50% **(Table 5.9a and Table 5.9b)**. We also stratified our validation cohort in different,

clinically distinct, sub-populations and observed that performance was maintained across all sub-populations with reliable NPV for advanced fibrosis over 74% **(Tables 5.10 and 5.11)**.

Table 5.9a
Predictive values of cut-off for different prevalence of advanced fibrosis (F>3);
"Rule in" advanced fibrosis

Combined Cohort (n=449)										
	FIBC3>-0.4		**ABC3D>3**		**FIB4>2.67**		**NFS>0.676**		**ADAPT >6.3**	
Prevalence of significant Fibrosis (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
5	15	99	15	98	19	96	22	96	14	98
10	27	97	26	96	33	92	38	92	26	96
15	37	95	36	94	44	87	49	88	36	94
20	46	93	45	91	52	83	57	84	45	92
25	53	91	52	89	59	79	64	80	52	90
30	59	89	58	86	65	74	70	75	58	87
35	65	87	63	83	70	69	74	71	63	85
40	69	84	68	80	75	65	78	66	68	82
45	74	81	73	77	78	60	82	61	72	78
50	77	78	76	73	81	55	84	57	76	75

Table 5.9b
Predictive values of cut-off for different prevalence of mild fibrosis (F≤2);
"Rule in" mild fibrosis

Combined Cohort (n=449)										
	FIBC3<-0.4		**ABC3D <3**		**FIB 4<1.3**		**NFS<-1.433**		**ADAPT<6.3**	
Prevalence of mild Fibrosis (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
5	25	99	12	98	11	98	14	98	12	97
10	42	97	23	97	21	95	26	95	22	94
15	53	96	32	95	30	93	36	92	31	91
20	62	94	40	93	38	90	45	90	39	88
25	68	92	47	91	46	87	51	87	46	85
30	73	90	53	88	51	84	57	84	52	81
35	78	88	59	86	57	81	63	80	58	78
40	81	85	64	83	62	78	68	76	63	74
45	84	82	69	80	67	74	72	73	68	70
50	87	79	73	76	71	69	76	68	72	65

Table 5.10: Performance of FIBC3 across sub-populations

Performance of FIBC3 across sub-populations (cut off >-0.4) in Validation Cohort n=298
Prevalence of advanced fibrosis 0.36

	Validation Cohort	'Normal' ALT<40U/l	'High' ALT>40U/l	Diabetic	Non-Diabetic	Male	Female	BMI <25	BMI>25
N	298	70	228	142	156	169	129	26	272
N with F3-F4	107	20	87	76	31	51	56	4	103
AUROC	0.84	0.92	0.81	0.83	0.72	0.84	0.81	0.88	0.82
Sensitivity	74	90	71	91	35	73	77	25	77
Specificity	75	72	77	41	94	77	73	96	73
PPV	63	56	65	64	58	58	68	50	63
NPV	84	94	81	79	85	87	80	88	84
Accuracy	77	77	75	68	82	76	74	85	74

Table 5.11 Performance of ABC3D across sub-populations

Performance of ABC3D across sub-populations (cut off >3) in Validation Cohort n=298
Prevalence of advanced fibrosis 0.36

	Validation Cohort	'Normal' ALT<40U/l	'High' ALT>40U/l	Diabetic	Non-Diabetics	Male	Female	BMI <25	BMI>25
N	298	70	228	142	156	169	129	26	272
N with F3-F4	107	20	87	76	31	51	56	4	103
AUROC	0.81	0.91	0.79	0.76	0.77	0.83	0.77	0.88	0.79
Sensitivity	64	85	64	91	13	67	69	25	70
Specificity	76	76	75	30	99	78	71	96	73
PPV	61	59	62	60	80	57	65	50	61
NPV	79	93	77	74	82	84	75	88	80
Accuracy	72	79	71	63	82	75	71	85	72

5.3.6. Performance of PROC3, FIBC3 and ABC3D as pre-screening tools prior to liver biopsy to support clinical trial recruitment

As there is also a need for tools to assist in pre-screening patients for clinical trials in NASH, the performance of PROC3 was modelled as pre-screening tools for entry into clinical trials of fibrosing steatohepatitis. Two target populations were modelled: (i) "tdNASH", defined as NAS ≥4 with at least 1 point each for steatosis, hepatocyte ballooning and hepatic inflammation and fibrosis stage ≥F2; and (ii) "tdNASH-Cirrhosis", defined as above but with fibrosis stage F4. For tdNASH, a PROC3 level >14.5ng/ml had an AUROC of 0.68 (sensitivity 59%, specificity 69%, accuracy 64%). This was replicated in the validation cohort (n=298), AUROC = 0.76, sensitivity 70%, specificity 68%, accuracy 69%. Similarly, a PROC3 level >16.5ng/ml identified tdNASH-Cirrhosis with an AUROC of 0.68 (sensitivity 74%, specificity 67%, accuracy 68%). This

was replicated in the validation cohort (n=298), AUROC = 0.76, sensitivity 76%, specificity 61%, accuracy 63% **(Table 5.3)**. Assessing the FIBC3 and ABC3D scores in the complete cohort (n=449), the results are shown in **Table 5.12**. In general, tests incorporating PROC3 performed well. The most accurate test for the detection of tdNASH was FIBC3>-0.4 (71%). The availability of data from the currently recruiting Phase 2/3 clinical trials will be informative to further validate these findings.

Table 5.12 Performance of fibrosis indices as pre-screening tools prior to liver biopsy						
Fibrosis Indices in Clinical Trial Settings (n=449)						
***tdNASH F2-F4 Detection**						
	PPV	**NPV**	**Sensitivity**	**Specificity**	**Accuracy**	**Number missed cases**
AAR>0.8	59	55	44	70	57	126
APRI>1.5	79	52	10	97	53	203
BARD>2	61	66	72	54	63	63
FIB4>2.67	76	53	17	95	56	187
NFS>0.676	78	54	20	94	57	180
ADAPT>6.3	73	67	61	77	69	87
FIBC3 >-0.4	74	68	64	77	71	81
ABC3D >3	73	66	59	78	69	92
***** tdNASH F3-F4**						
AAR>0.8	**25**	**81**	**44**	**65**	60	53
APRI>1.5	18	79	43	94	75	89
BARD>2	27	87	74	45	51	24
FIB4>2.67	22	79	12	89	73	83
NFS>0.676	26	80	16	88	73	79
ADAPT>6.3	32	87	65	64	64	33
FIBC3 >-0.4	32	88	67	63	64	31
ABC3D >3	31	86	61	65	64	37
****** tdNASH Cirrhosis (F4)**						
AAR>0.8	**20**	**92**	**61**	**66**	**65**	22
APRI>1.5	46	90	23	96	87	43
BARD>2	18	95	84	45	50	9
FIB4>2.67	40	91	36	92	85	36
NFS>0.676	40	92	41	91	85	33
ADAPT>6.3	25	97	86	64	67	8
FIBC3 >-0.4	27	98	93	64	67	4
ABC3D >3	26	97	86	66	68	8

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5.3.7. ABC3D to improve the accuracy of NFS and FIB4 Scores

Although FIB4 and NFS are useful, the use of two cut-off thresholds lead to indeterminate readings where they fail to classify a substantial proportion of patients. For each diagnostic test a method of sequential testing was employed by applying the low and high cut-off values. The residual cohort of NAFLD patients with intermediate scores were then subject to the ABC3D diagnostic algorithm to detect cases of advanced fibrosis **(Table 5.13 and Table 5.14).** With the application of sequential testing, the accuracy improved for the NFS from 52 to 70% in cases involving indeterminate FIB4 scores and from 54% to 77% in the case involving indeterminate NFS scores.

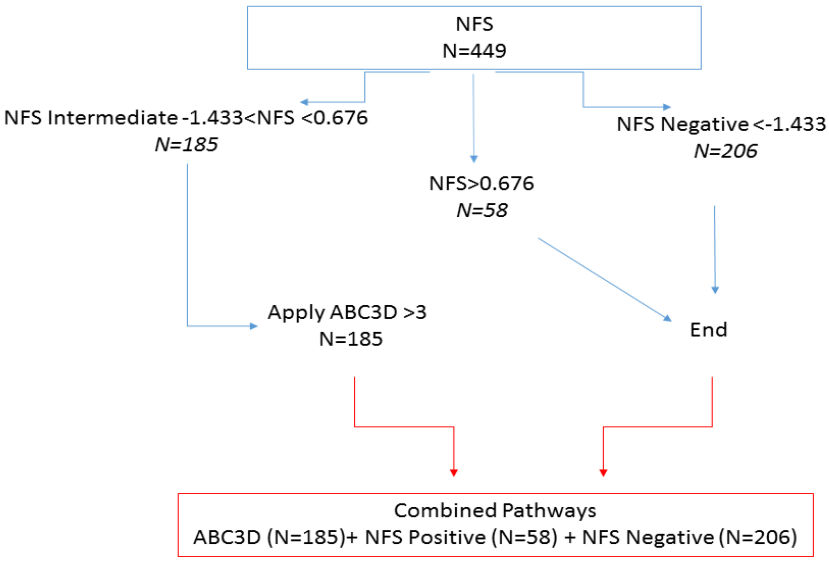


Table 5.13: Sequential testing NFS and ABC3D

(n=449)	TP	TN	FP	FN	Total	PPV	NPV	Sen	Spec	Accuracy
Negative (n=206) NFS<-1.433	0	175	0	31	206	0	85	0	100	85
Positive (n=258) NFS > 0.676	45	0	13	0	58	78	0	100	0	78
Intermediate (n=185) **-1.433<NFS <0.676 Application ADC3D>3 to detect advanced fibrosis**	69	58	36	22	185	66	73	76	62	69
Combined (n=449)	114	233	49	53	449	70	81	68	83	77
NFS applied to total cohort (assuming intermediate readings as a combination of FN and FP) **Apply calculation for accuracy =** (TP+TN)/(TP+TN+FP+FN) = 45+175/ (45+175+185) = 54%										

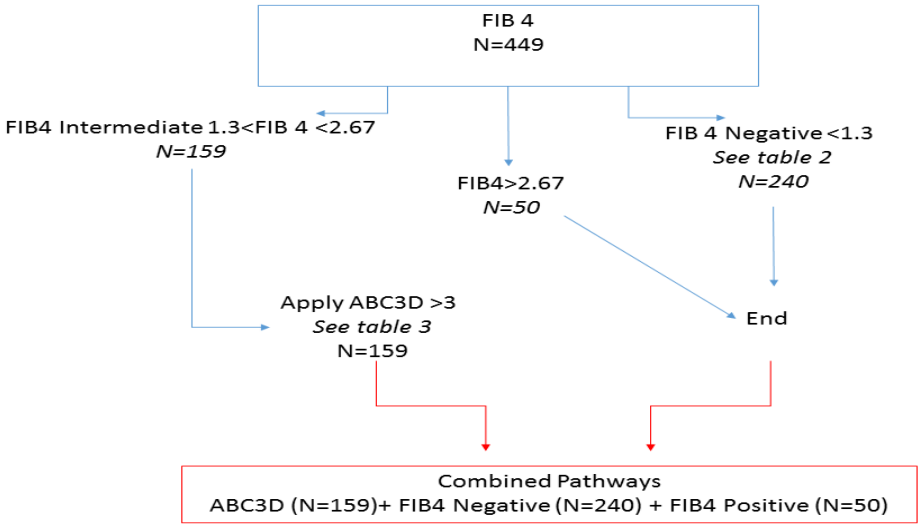


Table 5.14: Sequential testing- FIB4 and ABC3D

(n=449)	TP	TN	FP	FN	Total	PPV	NPV	Sen	Spec	Accuracy
Negative (n=240) FIB4<1.3 To detect mild fibrosis	0	194	0	46	240	0	81	0	100	81
Positive (n=50) FIB4> 2.67 To detect severe fibrosis	36	0	14	0	50	72	0	42	0	36
Intermediate (n=159) **1.3<FIB 4 <2.67 Application ADC3D>3 to detect severe fibrosis**	65	54	20	20	159	77	83	76	73	75
Combined (n=449)	101	248	34	116	449	75	68	47	88	70

FIB 4 applied to total cohort (assuming intermediate readings as a combination of FN and FP)
Apply calculation for accuracy = (TP+TN)/(TP+TN+FP+FN)= 194+36/(194+36+209)= 52%

5.3.8. Development of PNPLA3 and PROC3 Diagnostic model

The G allele in the forward strand of s738409 C/G -a variant nonsynonymous single nucleotide polymorphism (SNP) of PNPLA3 (patatin-like phospholipase domain containing 3) is a robust genetic marker of disease severity. The value of adding PNPLA3 to a diagnostic panel including PROC3 to predict advanced fibrosis was investigated. PNPLA3 genotype was available on 358 patients. This cohort was subdivided into a validation (n=234) and discovery cohort (n=124) as per **Figure 5.4**. **Table 5.15** displays the clinico-demograpic details of the study population. A model was generated incorporating the PNPLA3 genotype (allelic dominant) as outlined in **Table 5.16.**

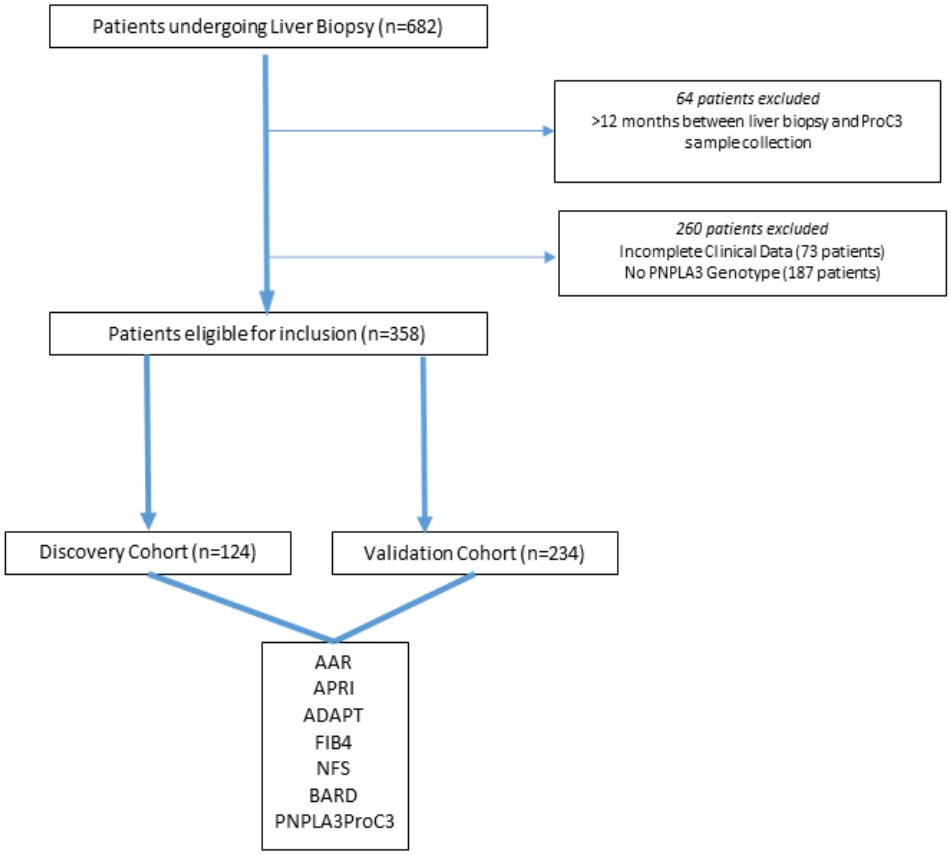


Figure 5.4: Patient flow

Table 5.15 Baseline Demographic and Clinical characteristics of participants				**Discovery Vs Validation Cohort**
Variable	**All Patients (n=358)**	**Discover Cohort (n=124)**	**Validation Group (n=234)**	**P-Value**
Age (years)	51+/-13	51+/-12	51.8+/-13	*0.206*
Gender (male)	211(59%)	72(58 %)	139(59 %)	*0.807*
BMI (Kg/m^2)	32.3+/-6.4	32.6+/-6.1	32.2+/-6.6	*0.383*
T2DM	169(47%)	199(47%)	85(46%)	*0.918*
ALT (U/l)	71+/-43	68+/-43	73+/-43	*0.455*
High ALT (>40U/l)	272(76%)	96(77%)	176(75%)	*0.642*
AST (U/l)	48+/-27	48+/-28	48+/-26	*0.597*
Albumin (g/dl)	44+/-4	44+/-4	44+/-4	*0.512*
Platelets (X10^9/l)	228+/-71	223+/-62	230+/-75	*0.432*
Cholesterol (mg/dl)	7+/-12	5+/-2	7.2+/-14	*0.238*
Triglycerides (mg/dl)	3.5+/-16	4.4+/-20	3.0+/-13	*0.833*
Collagen Pro-C3(ng/ml)	19.3+/-15	18.5+/-15	19.8+/-15	*0.252*
Collagen Pro-C6(ng/ml)	9.9+/-4.7	9.7+/-5.1	10.0+/-4.5	*0.183*
P4NP7S (ng/ml)	272+/-148	265+/-150	275+/-148	*0.521*
C4M2 (ng/ml)	27.2+/-10	27.4+/-11	27.0+/-9.8	*0.972*
C3M (ng/ml)	11.6+/-5	11.5+/-4.6	11.6+/-4.5	*0.819*
Fibrosis Stage (0/1/2/3/4)	70/73/75/86/54	19/29/28/32/16	51/44/47/54/38	*0.454*
Steatosis 0/1/2/3	6/115/134/94	3/38/43/38	3/77/91/56	*0.475*
Ballooning 0/1/2	96/148/103	27/55/40	69/93/63	*0.228*
Lobular Inflammation 0/1/2/3	34/175/117/21	15/53/43/11	19/122/74/10	*0.117*
NAS	4+/-2	5+/-2	4+/-2	*0.510*
FIB4	1.54+/-1.10	1.56+/-1.16	1.53+/-1.07	*0.865*
AAR	0.76+/-0.32	0.79+/-0.35	0.75+/-0.30	*0.287*

NAFLD Fibrosis Score	-1.351+/-1.84	-1.257+/-1.687	-1.400+/-1.919	*0.080*
APRI	0.698+/-0.50	0.71+/-0.57	0.69+/-0.45	*0.938*
ADAPT Score	6.38+/-2.29	6.25+/-2.3	6.45+/-2.31	*0.396*
BARD Score	2+/-1	2+/-1	2+/-1	*0.142*
Centrally Read Biopsies	226(63 %)	76 (61 %)	150(64 %)	0.562
PNPLA3 CC/GG/GC	125/65/168	41/26/57	84/39/111	0.458
PNPLA3 G-Dominant	233 (65%)	83 (67%)	150 (64%)	0.593

^The table shows the mean ± SD for continuous variables, number (%) for binary variables, and number per group for categorical variables

NAS= NAFLD Activity Score; *sNASH =steatosis with hepatocyte ballooning ± inflammation ± fibrosis, or steatosis with ≥ stage F2 fibrosis **tdNASH = steatosis with a NAS ≥4 with at least 1 point each for steatosis, hepatocyte ballooning and hepatic inflammation and stage ≥ stage F1 fibrosis ***fNASH = steatosis with least 1 point for hepatocyte ballooning and hepatic inflammation.

T-Test/Mann Whitney was used to test for significant differences within continuous variables and Chi-Square test was used for categorical variables.

BMI= Body mass index; T2DM= Type 2 Diabetes Mellitus; ALT = Alanine Aminotransferase; AST= Aspartate aminotransferase FIB4= Fibrosis-4 Index; AAR= AST to ALT ratio; APRI= AST to platelet ratio index. ADAPT = Algorithm including Age, Diabetes, Pro-C3 and platelet count. BARD = BMI, AST/ALT ratio, Diabetes

Table 5.16 Variables Associated with the Presence of Advanced Fibrosis (stage 3-4) in the Discovery Cohort (n=124) Prevalence advanced fibrosis (0.39)

	Univariate			Adjusted (Multivariate)		
Variable	**Odds Ratio**	**95% CI**	**p-value**	**Odds Ratio**	**95% CI**	**p-value**
Age	1.063	1.026-1.102	0.001			
Gender	0.671	0.323-1.394	0.284			
BMI	1.121	1.047-1.2	0.001	1.111	1.011-1.222	0.029
T2DM	8.256	3.579-19.047	<0.0001	9.850	3.283-29.551	<0.0001
ALT	1.008	0.999-1.017	0.088			
AST	1.025	1.008-1.043	0.003	1.016	0.997-1.034	0.096
Albumin	0.900	0.811-0.998	0.045			
Platelets	0.989	0.982-0.996	0.002	0.989	0.980-0.997	0.009
Cholesterol	1.023	0.863-1.213	0.792			
Triglycerides	0.986	0.945-1.029	0.508			
PROC3	1.076	1.033-1.121	<0.0001	1.071	1.014-1.132	0.014
AST-ALT Ratio	2.143	0.749-6.132	0.155			
PNPLA3 G Positive	0.842	0.392-1.807	0.658	0.311	0.101-0.965	0.043
Formula: -3.920+(-1.167*PNPLA3genotype G positive) + (0.106*BMI)+(2.287*T2DM)+(0.016*AST)+ (-0.011*Platelets)+(0.069*ProC3)						

The optimal threshold value of >-1.04 for the new diagnostic model was chosen by maximizing the Youden index for the corresponding ROC curve (sensitivity 94%, specificity 68%, PPV 65% and NPV 95%). The predicted diagnostic accuracy as per AUROC curves for the PROC3PNPLA3 model in the discovery, validation and combined cohorts was 0.90, 0.85, 0.86 respectively, comparable to the ADAPT model (0.85,0.88,0.87), FIBC3 (0.87,0.87,0.87) and ABC3D (0.78, 0.80, 0.79) ***(Table 5.17)***. In the validation cohort, PROC3PNPLA3 model performance was similar to FIBC3, ADAPT and ABC3D with accuracies for the detection of

advanced fibrosis measured at 76% versus 79%, 77% and 74% respectively ***(Table 5.18)***. The PROC3PNPLA3 model correctly staged advanced fibrosis in 71% of cases performing inferiorly to all PROC3 based models; FIBC3 (79%), ABC3D (74%) and ADAPT (77%). ***(Table 5.18)*** In the validation cohort comparison of AUROC curves (using DeLong, DeLong, and Clarke Pearson method) for the detection of advanced fibrosis showed no significant differences; PROC3PNPLA3 versus ABC3D (p= 0.0604); PROC3PNPLA3 versus FIBC3 (p= 0.2140) and PROC3PNPLA3 versus ADAPT (p= 0.1631), meaning that the addition of genetic information to a fibrosis detection model involving PROC3 did not improve the diagnostic accuracy therefore its inclusion cannot be recommended.

Table 5.17 Diagnostic Accuracy of Non-invasive Tests in Detecting Histologic Stage F3–F4: Prevalence advanced fibrosis in combined (0.39) Discovery (0.39) and validation cohort (0.39)

Combined Cohort (n=358)			**Discovery Cohort (n=124)**		**Validation Cohort (n=234)**	
Non-invasive Test	**AUROC**	**95% CI**	**AUROC**	**95% CI**	**AUROC**	**95% CI**
AAR	0.69	0.6356-0.7450	0.66	0.5656-0.7562	0.71	0.6385-0.7730
APRI	0.75	0.6939-0.8001	0.79	0.7084-0.8722	0.72	0.6542-0.7922
BARD	0.75	0.6937-0.7973	0.73	0.6413-0.8201	0.75	0.6899-0.8172
FIB4	0.78	0.7354-0.8316	0.79	0.7085-0.8650	0.78	0.7224-0.8448
NFS	0.82	0.7736-0.8658	0.83	0.7545-0.9026	0.81	0.7547-0.8724
ADAPT	0.87	0.8302-0.9062	0.85	0.7815-0.9154	0.88	0.8339-0.9260
PROC3PNPLA3	0.86	0.8242-0.9023	0.90	0.8438-0.9503	0.85	0.7958-0.8998
PROC3	0.77	0.7215-0.8227	0.74	0.6473-0.8288	0.79	0.7311-0.8524
FIBC3	0.87	0.8291-0.9077	0.87	0.8071-0.9331	0.87	0.8151-0.9164
ABC3D	0.79	0.7487-0.8410	0.78	0.6934-0.8579	0.80	0.7491-0.8605

Table 5.18 Optimal Cut-off values for the detection of advanced fibrosis (>F3) as per Youden Index (YI) derived in Discovery Cohort (Prevalence 0.39, n=124); Application in Validation Cohort (Prevalence 0.39, n=234)

Panel	AUC	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy
PROC3PNPLA3	0.90	>-1.04	94	68	65%	95%	78%
	Cut-off	**Sensitivity**		**Specificity**	**PPV**	**NPV**	**Accuracy**
AAR	>0.8	49%		76%	57%	70%	65%
APRI	>1.5	12%		98%	79%	63%	64%
BARD	≥2	79%		58%	55%	81%	66%
FIB4	>2.67	22%		97%	83%	66%	68%
NFS	>0.676	30%		96%	85%	68%	70%
ADAPT	>6.3	76%		78%	69%	83%	77%
FIBC3	>-0.4	80%		79%	71%	86%	79%
ABC3D	>3	74%		74%	65%	81%	74%
PROC3PNPLA3	>-1.04	83%		71%	65%	86%	76%

Table 5.19 Validation cohort divided into mild and severe fibrosis (Prevalence 0.39, n=234)

	F0-2 'Rule out' advanced fibrosis				**F3-4 'Rule in' advanced in severe**		
	Correctly identified	Indeterminate	Incorrectly Identified		Correctly identified	Indeterminate	Incorrectly Identified
N=142				N=92			
AAR AAR<0.8	107/142 75%		35/142 25%	**AAR AAR>0.8**	45/92 49%		47/92 51%
APRI APRI<0.5	83/142 58%	56/142 39%	3/142 3%	**APRI APRI>1.5**	11/92 12%	59/92 64%	22/92 24%
BARD BARD<2	82/142 58%		60/142 42%	**BARD BARD≥2**	73 79%		19 21%
FIB4 FIB4<1.3	101/142 71%	37/142 26%	4/142 3%	**FIB4 FIB4>2.67**	20/92 22%	49/92 53%	23/92 25%
NFS NFS<-1.433	85/142 60%	42/142 30%	15/142 10%	**NFS NFS>0.676**	28/92 30%	47/92 51%	17/92 19%
ADAPT ADAPT<6.3	110/142 77%		32/142 23%	**ADAPT ADAPT>6.3**	70/92 76%		22/92 24%
PROC3PNPLA3 <-1.04	101/142 71%		41/142 29%	**PROC3PNPLA3 >-1.04**	76/92 83%		16/92 17%
FIBC3 FIBC3<-0.4	112/142 79%		30/142 21%	**FIBC3 FIBC3>-0.4**	74/92 80%		18/92 20%
ABC3D ABC3D>3	105/142 74%		37/142 26%	**ABC3D ABC3D<3**	68/92 74%		24/92 26%

5.4. DISCUSSION

NAFLD has an estimated global prevalence of 25%, which is predicted to rise internationally (8, 236, 530). Associated mortality is directly proportional to fibrosis stage, with patients at ≥F3 being at highest risk (128). Current non-invasive tests are suboptimal; therefore, there is a clear need for better diagnostic biomarkers to detect advanced fibrosis. Such tests could potentially aid diagnosis and risk stratification, as well as facilitate clinical trial pre-screening to reduce screen failure rates; all of which fall within the BEST *diagnostic* context of use (531).

5.4.1. Limitations of reference standard

At present, the reference standard to assess severity of NAFLD is histological, using the semi-quantitative NASH CRN system (45). However, it is generally accepted that inter- and intra-observer variability, and sampling error due to variability in the extent of fibrosis within the liver, may impair the accuracy and reproducibility of these histological assessments (45, 126, 532). This implies a paradox that makes addressing the need for biomarkers all the more challenging: the histological reference standard, against which a biomarker is assessed, is inherently imperfect and unable to produce a completely error-free classification with respect to the presence or absence, or severity, of the target condition. Semi-quantitative histological grading conflates anatomical distribution of fibrosis with extent and imposes discrete categorical staging bins on what are continuous variables like collagen deposition (45). This inevitably leads to discrepancies due to inter- and intra-observer judgement, especially at the margins. It also blunts sensitivity as semi-quantitative grades fail to recognise modest differences in severity that do not transition across predefined but arbitrary categorical boundaries. This phenomenon is well illustrated by the breadth of disease that is encompassed by stage F3 fibrosis in the NASH CRN classification (45) where histological portal-portal, central-central and/or portal-central bridging is the defining feature, yet no weight is given to density of collagen deposition or the number of “bridging” septae. The situation where an imperfect reference standard is used in place of a perfect standard, introduces “imperfect gold standard bias”. This means that the performance of the new test may be under- or over-estimated and, even if it is in reality a better measure of disease, it never has the potential to generate an AUROC >0.90 (533). Although not unique to liver histopathology, such situations are methodologically challenging to address (534).

Cognisant of these challenges, this study reports measurement of PROC3 levels in a large international cohort and incorporate this measure into novel diagnostic models that outperform numerous previously described blood-based tests that detect advanced fibrosis (164, 165, 174, 177, 506-508).

5.4.2. Utility of PROC3 as a single diagnostic biomarker

Although isolated parameters seldom exhibit an adequate level of diagnostic accuracy and are unlikely to be a surrogate for the complex diagnostic information provided by liver biopsy, we assessed how PROC3 performed in this context of use, encouraged by its competitive performance described in ***chapter 5***. PROC3 performed moderately as a biomarker of advanced fibrosis, comparable to simple panels such as FIB4. Similarly, when used to screen patients for clinical trial recruitment, PROC3 accurately identified 65% of cases that were histologically eligible for current Phase III trial recruitment (NASH with significant fibrosis). This moderate performance as a diagnostic biomarker may partially be explained by the biological process that generates PROC3 during collagen deposition, implying that PROC3 is most sensitive to active fibrogenesis rather than static collagen accumulation. Supporting this view, preliminary evidence suggests that PROC3 may aid the evaluation of patients with active collagen turnover (484). In the present study, the value of the tool as a prognostic test that could be used to enrich studies for cases at greatest risk of subsequent disease progression or to monitor change in disease activity was not assessed. The PROC3 assay is robust in that it is directed toward a well-defined epitope that is generated during active fibrogenesis. This is an attractive concept for a pre-screening clinical trial biomarker where this biomarker has the potential to reliably measure the amount of disease activity based on the dynamics of fibrogenesis (498, 535).

5.4.3. FIBC3 and ABC3D performance for risk stratification of fibrosing steatohepatitis

In light of the moderate performance of PROC3 as a single diagnostic biomarker, its value as part of a non-invasive fibrosis panel composed of routinely measured clinical and laboratory variables was assessed. The objective was to determine if the panel performance was enhanced by inclusion of a single biomarker of fibrogenesis, PROC3. The development and validation of FIBC3 was reported. Whilst not the first panel to incorporate these

components, many of which are used within ADAPT (479), the current study benefits from detailed development and validation in a large, international patient cohort where careful harmonization of histological practice, coupled with central reviewing of biopsies, has been undertaken to minimize the potential impact of an imperfect reference standard. Overall, a FIBC3 threshold of >-0.4 correctly identified fibrosis status in 77% of patients in the total cohort. However, the diagnostic accuracy of ABC3D, a simplified panel, better adapted for use in clinical practice (at the bedside) rivalled this model with an accuracy of 75% and performed equivalently when assessed across different clinical sub-populations and consistently outperformed all other routinely used scores to which it has been compared. Thus, in contrast to FIB4, NFS or the PROC3 based ADAPT score which require more complex formulas, this simple model can be easily calculated by summing 5 easy to assess clinical items, removing the need to access a web-based calculator or App to aid patient risk stratification. In order to maximise sensitivity and specificity, "simple scores" such as FIB4 and NFS employ two cut-off thresholds and so leave a large "indeterminate" group that cannot be accurately stratified. FIBC3 and ABC3D are superior in that they do not suffer from this limitation and so are less prone to residual diagnostic uncertainty. To illustrate this point, this study employed sequential testing incorporating ABC3D demonstrating its clinical utility in cases of indeterminate FIB4 scores and NFS where the accuracies improve from 52% to 70% and 54% to 77% respectively with sequential testing.

In the validation cohort, FIBC3 performed best correctly identifying 75% of patients, with ABC3D more or less equivalent correctly identifying 72% of patients. In the full cohort of 449 patients, the FIBC3 model identified 254 patients as not having advanced fibrosis (at a threshold of less than -0.4) of which 217 were correctly classified. Therefore, in this "low-risk cohort" the FIBC3 model could have correctly avoided a liver biopsy in 85% of patients. Applying the same analysis to ABC3D, 267 patients were identified as 'low-risk' (score$\leq$3). In this cohort, 219 patients were correctly staged thus potentially correctly avoiding biopsies in 82% of cases. Complex fibrosis panels also exist. They include markers of matrix turnover, such as the Enhanced Liver Fibrosis (ELF) panel (508). However, a recent meta-analysis has reported ELF^{TM} and NFS had very similar AUCs (536). Extrapolating this observation to this

data, would imply that FIBC3/ABC3D (like the NFS) had comparable, if not better, diagnostic value than the more complex Fibrotest™ and ELF™.

5.4.4. Potential to use ABC3D in Primary Care

The point performance of diagnostic tests in terms of PPV/NPV are affected by pre-test probability, which reflects the prevalence of disease in a specific clinical setting. The prevalence of advanced fibrosis in the current study cohort was 37% which is much higher than what is expected in a primary care setting. Indeed, population data, albeit limited, have found that 5.6% of the Dutch population have clinically significant fibrosis based on a VCTE liver stiffness >8kPa (537). Similarly, based on VCTE thresholds ≥6.8, ≥8.0, and ≥9.0 kPa prevalence estimates in the Spanish population were 9.0%, 5.8%, and 3.6% respectively (538). These levels contrast sharply to a tertiary referral centre where the prevalence of advanced liver disease is often well in excess of 10%, and frequently nearer 30% (539-541). To model performance across a range of settings, we calculated PPV and NPV for prevalence levels of advanced fibrosis from 5-50%. The NPV for both FIBC3 and ABC3D were similar across a prevalence range of 5-15% and in excess of 90%. To explore performance of the models in specific patient subgroups, we split the cohort by gender, diabetes status, BMI, and patients with elevated or normal ALT levels. FIBC3 and ABC3D maintained high NPV in all subgroups, although sensitivity was lower in patients with a BMI<25 and non-diabetics.

5.4.5. PROC3PNPLA3 Model

The association of genetic variability in patatin-like phospholipase domain-containing protein 3 (PNPLA3) with histological severity of non-alcoholic fatty liver disease has been widely reported (542). However, in keeping with previous studies, the development of a model incorporating a genetic marker did not improve the diagnostic accuracy of PROC3 based models, with the generation of similar ROC curves for the diagnosis of advanced fibrosis. (***AUROC*** PROC3PNPLA3 versus FIBC3, p=0.2140; versus ABC3D, p=0.0604; versus ADAPT p=0.1631). One could speculate that PROC3 plus the routinely available clinical data already reflect the influence of this SNP on liver fibrosis stage. Possible explanations arise from the fact that NAFLD is a multifactorial disease where genetic, metabolic risk factors and environmental factors play a role in causation, thus PNPLA3 as a single risk factor may

not bear a substantial effect on NAFLD disease severity (542). This finding resembles other examples from the literature showing that the incorporation of 16 SNPs improved the prediction of type 2 diabetes by only 1% if added to data on family history, BMI, liver enzymes, smoking status, and reduced insulin action (543). A further example was observed in the derivation of the NAFLD fat score/equation where knowledge of the PNPLA3 phenotype improved the accuracy of the identification of NAFLD <1% when added to the Metabolic syndrome profile, fasting serum insulin, AST and AST/ALT measurements (520)

5.4.6. Study strengths & Limitations

Study cohort FIBC3 and ABC3D were developed using an international cohort of well-characterized, untreated NAFLD patients covering a wide spectrum of disease severity.

Reference standard Liver biopsies were read by expert histopathologists that belong to the EPoS Pathology consortium, a group that undertook extensive harmonisation procedures for NAFLD pathological assessment and demonstrated high kappa-value reproducibility (126). Moreover, half of the biopsies across all sites were assessed centrally. While this certainly reduces the reader-related variability, it is still dependent on limitations intrinsic to histological classifications such as the semi-quantitative nature of fibrosis scoring and on sampling variability of the procedure. These limitations are common to all biomarkers that use biopsy as the reference standard. To minimize the effects of inter-observer variability in fibrosis staging half the cohort across all centres had centrally reviewed liver biopsies confirming high inter-observer agreement. Although we have taken measures to minimize inter-observer variability in the histological scoring, and concordance between liver pathologists was very good, an element of variability cannot be fully excluded. We also acknowledge that percutaneous liver biopsy is prone to sampling error leading to miss-staging of disease severity. However, the key limitation, which is common to all biomarker studies that rely on histology, relates to the nature of the semi-quantitative scoring systems and how this conflates histological localisation of fibrosis and extent of collagen deposition.

Diagnostic model parameters this diagnostic model consists of readily available clinical and laboratory variables that are routinely determined in patients with NAFLD in outpatient

appointments. PROC3 levels were also measured in a central College of American Pathologists (CAP) certified lab by staff blinded to the clinical data and results sent to a separate, independent centre for statistical analysis. Protein finger print technology has been developed to produce a reliable assay for PROC3 measurement (513). Our model, in comparison to previous complex biomarker panels (e.g. ELF™, Fibrotest™) includes only one variable that is not routinely measured in a clinical setting. It is regrettable that this study was unable to benchmark PROC3 against these commonly employed ECM biomarker tests because of insufficient serum.

Statistics AUROCs are not optimal as a means for assessing diagnostic accuracy. ROC curves attribute equal weight to false positives and false negatives and do not provide information on predictive values, which are of more value in a clinical setting (544). In addition to this, the relevance of ROC curves is sub-optimal given its dependence on liver biopsy as a reference standard.

Clinical application of model the availability of Apps on smart phones facilitates conduct of complex calculations (FIBC3) but clinicians would consider an intuitive model that can be easily remembered, without recourse to an electronic calculator, to have value. The ABC3D model is valuable because recourse to an online app in a busy clinical setting may sometimes not be preferable. ABC3D can be used, using simple mental arithmetic in clinic. In this respect, the comparison of ABC3D vs. FIBC3 is akin to a comparison between Child-Pugh score vs. MELD – both have great utility, but one is much simpler to capture than the other.

Similarities between FIBC3, ABC3D and ADAPT. As is apparent from the data presented, the overall differences between FIBC3, ABC3D and ADAPT are marginal, although FIBC3 appears to have the edge in the current study. In looking to compare diagnostic accuracies, the literature suggests that rather than looking for p-values for isolated accuracy readings (based on testing the classification error) one should look at more robust measurements such as AUROC. As might be expected from the data presented in ***Table 6.6***, comparison of AUROC curves for FIBC3 and ABC3D for the detection of advanced fibrosis were not significantly different (p= 0.1422, using DeLong, DeLong, and ClarkePearson method), nor

were FIBC3 from ADAPT (p= 0.1859), meaning they are numerically superior but comparable diagnostic accuracies. The adjusted AUROC data indicate similar or slightly superior performance for FIBC3 and ABC3D vs. ADAPT. The key differentiator between ADAPT and ABC3D is ease of use. ***Table 6.7*** demonstrates that FIBC3 has superior Sensitivity and Accuracy with similar specificity to ADAPT: FIBC3 correctly identifies more cases. ABC3D had greater Specificity and similar accuracy to ADAPT. It should be noted however that the differences in NVP are marginal, just 1% between ADAPT and ABC3D. Ultimately the merits of ABC3D vs. ADAPT lie in ease of use vs. only a marginal drop in performance. Where an electronic calculator is to be used, FIBC3 appears to have marginally better performance than ADAPT across all measures ***(Table 6.12).***

This study provides much needed independent validation of the performance of ADAPT. The variables that we have used in FIBC3 and ABC3D (Age, BMI, platelet Count, diabetes and PRO-C3) do partially overlap with those used in ADAPT (age, presence of diabetes, PROC3, and platelet count). However, this should not be considered as a weakness. Indeed, it should be noted that the same criticism could be said of ADAPT in comparison to the preceding "NAFLD Fibrosis Score" – age, presence of diabetes and platelet count are common to many scores used in NAFLD as they are important indicators of disease progression risk. All variables used in FIBC3 and ABC3D were derived as statistically significant in our data set, making their inclusion robust, and are widely reported in the literature as having an association with advanced fibrosis.

Overlap with current PROC3 publications there has been a recent increase of PROC3 data in the literature. Two current prominent studies include work by Caussy et al and Luo et al (465, 545). However, on review, each examine very different aspects of PROC3 to those that are addressed in this study. The former is primarily a genetic-trait association study with some cross validation against another experimental biomarker (MR Elastography) as a reference standard. The latter explores changes in PRO-C3 levels with time, i.e. the 'monitoring' context of use. Both use substantially smaller cohorts with markedly differing routes of patient ascertainment to the current study. Arguably, the greatest similarity is with the work by Daniels et al however, here too there are important differences. The current study once again employs a larger cohort of patients and, unlike Daniels et al, where

all biopsies were read by pathologists at the local recruiting centres, over half of the biopsies used in the current study were centrally read and all pathologists had participated in a harmonisation procedure with documented high kappa value inter-observer agreement, making the conclusions more robust. In addition, the analysis of biomarker performance in the current study has been conducted by researchers fully independent of the biomarker manufacturer – biological samples were processed by staff that had no means of accessing clinical phenotype data and the statistical analysis has been conducted at an academic centre by staff that are not employees of the manufacturer. The current study is therefore the first study to implement such a robust methodological approach and that provides a truly independent analysis of PROC3 biomarker performance for the diagnostic context of use. Further value of the current study and additional novelty stems from (1) the development of both FIBC3 model and a simplified ABC3D clinical tool that does not require the use of an online calculator/app; (2) the much needed independent validation of ADAPT; and (3), a comprehensive supplementary dataset that contains information of substantial value to future researchers wishing to implement the use of PROC3 in subsequent clinical studies as a pre-screening tool for therapeutic trials, or for use in clinical practice.

5.4.7. Future Directions

Guidelines for use if PROC3 is successfully translated from bench to bedside as a biomarker in line with FDA recommendations, it will be necessary to provide (1) an exact biomarker definition, with clarification of the aspect being measured in a biological context, (2) an evaluation of the exact utilisation of the biomarker in clinical trials with guidelines on its interpretation and decision based on a binary outcome. Another equally important area to develop will be to explore PROC3 as a biomarker for disease progression/regression to aid the identification of the most suitable candidates for clinical trial recruitment or treatment thus avoiding exposure to experimental treatments and reducing health care costs (546).

Combine with other collagen biomarkers A cirrhotic liver is collagen rich (containing 10 times more collagen than a physiologically normal liver) (130). Additional collagen biomarkers have been characterized representative of formation and degradation (PROC4, PROC6) (C3M, C4M) respectively. It is possible that combination biomarkers may allow us to

refine the findings in this study by developing a fingerprint marker of active fibrogenesis and thus indicate treatment responders.

Improvement in reference standard in biomarker development there is now mounting evidence that the inherent variation in liver biopsy makes this a suboptimal reference standard***.*** Advances in artificial intelligence (AI) in liver imaging may overcome inherent flaws in liver biopsy as a reference standard for biomarker development. A branch of machine learning, called "deep learning systems" (DLS) is based on a neural network modelled on the human brain. The multi-layered "convolutional neural networks" (CNNs) are the most popular type of DLS in the medical image analysis field (547) that operate by navigating the data space, classifying images and processing tasks with the fully connected layers performing high level reasoning before generation of the final output and have been applied to CT, Ultrasound shear wave elastography, and MRI images (548-550). In a recent study, a deep learning system was developed for portal venous phase CT images which outperformed the radiologist's interpretation, APRI and FIB4 for staging liver disease with AUROC of 0.96, 0.97 and 0.95 for the diagnosis of fibrosis (F2-F4), advanced fibrosis (F3-F4) and cirrhosis. Fibrosis assessments in this manner are non-invasive, adopt predefined accurate algorithms therefore are not subject to human error and provide a whole liver assessment therefore eliminating sampling error.

5.5. CONCLUSION

Both FIBC3 and ABC3D are simple indices including accessible routine laboratory tests and a single marker of collagen turnover. It has been shown that both can accurately differentiate mild to moderate fibrosis from bridging fibrosis and cirrhosis in patients with NAFLD. Given that the ABC3D model is much simpler to compute and can be done at the bedside, the ABC3D diagnostic index with validation has the potential to be widely used for the identification of patients with significant/active fibrosing steatohepatitis who should undergo specialized liver explorations, closer monitoring and possibly, specific therapies. FIBC3 and ABC3D may also be used as pre-screening tools for therapeutic trials, potentially helping to minimise histological severity-related screen failure rates however, this will require further prospective validation

CHAPTER 6.

PLASMA DNA METHYLATION AS A BIOMARKER FOR STRATIFICATION OF MILD AND SEVERE LIVER FIBROSIS IN NON-ALCOHOLIC FATTY LIVER DISEASE

6.1. INTRODUCTION

NAFLD is a paradigm for the complex disease trait; subtle inter-patient genetic variations and environment interact to determine disease phenotype and progression (52). Progress in our understanding of the pathogenesis, diagnosis and risk factors for progressive disease are urgently needed. Factors causative for NAFLD progression include genetic predisposition and epigenetic modifications that have been observed in both the nuclear and mitochondrial genome and represent areas with potential for biomarker development (232, 551-555). There is a body of literature to suggest that epigenetic marks can be modified by environmental factors such as alcohol consumption, diet and lifestyle and the dynamic nature of epigenetic modifications is also a promising biomarker characteristic (556, 557). Recently, biomarker development has been strongly influenced by the evolving implementation of high-throughput OMICs profiling of biological samples. However, integration of this knowledge into the healthcare system is both time and labour intensive.

6.1.1 Epigenetics

Epigenetic change is a biological phenomenon that is influenced by naturally occurring influences. Epigenetic modifications are both mitotically and transgenerationally heritable and may persist transgenerationally despite lack of continued exposure in subsequent generations (558-561). They include DNA methylation and histone/chromatin structure, which dictate the degree to which loci within the genome can be transcribed. Non-coding RNAs have also been implicated in almost every level of control of gene expression and protein function. There is substantial interplay between these distinct mechanisms, which combine to determine cellular and ultimately disease phenotype. As alluded to in the introduction, DNA methylation plays an important role in hepatic fibrogenesis (see chapter 1).

6.1.2. DNA methylation and NAFLD

A study examined differential DNA methylation in NAFLD in 69 247 CpG sites in liver biopsies from mild (F0-2) versus patients with advanced (F3-F4) fibrosis (216). The majority of Differentially Methylated Regions (DMRs) became hypomethylated with disease progression (76%), whereas 24% underwent hypermethylation (***Figure 6.0).*** Not surprisingly, many

genes that modulate wound healing responses were hypomethylated in advanced NAFLD, and pathway analysis confirmed that processes involved in fibrogenesis were induced suggesting that methylome-transcriptome interactions that occur in advanced NAFLD can modulate NAFLD outcomes (216). Other work has addressed the potential genetic and epigenetic cross-talk in NAFLD (562). Zeybel et al have reported that differences in methylation at genes implicated in fibrogenesis can stratify patients into stable mild fibrosis vs severe fibrosis (PPARα, PPARg, TGFβ1, Collagen 1A1 and PDGFα genes) (167). The characterization of such 'methylation signatures' allowed patient stratification to be performed according to disease severity. A key question remains as to whether NAFLD specific methylation information is ready to be implemented in a clinical setting.

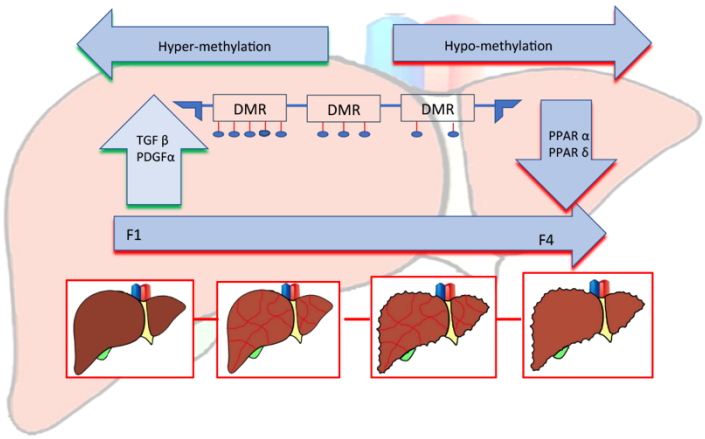


Figure 6.1: DNA methylome-transcriptome interactions that occur in advanced NAFLD can modulate NAFLD outcomes

With NAFLD fibrosis progression (F1-F4), multiple DMRs associated with genes involved with wound healing responses become hypo-methylated resulting in the induction of pathways associated with carcinogenesis and fibrogenesis.

6.1.3. Possibility of non-invasive liquid biomarker

NAFLD specific epigenetic signatures (with a direct effect on liver function) have been derived from both liver tissue and circulating cell free DNA (167, 168). The concept of ccfDNA as a 'liquid biopsy' has been discussed in the introduction (chapter 1). Small

fragments of DNA circulate in the peripheral circulation and are thought to originate from apoptotic cells and are thus representative of ongoing cell death (217) CcfDNA has been researched as a biomarker of NAFLD disease severity, however results have always been undermined by the lack of a definitive association between ccfDNA and NASH biology(168) It has been shown that plasma ccfDNA received a contribution from apoptotic hepatocytes (1%)- which has the potential for exploitation as a liver specific biomarker. Unfortunately, considerable analytical and technical challenges associated with ccfDNA present challenges for biomarker development (218).

In recent publications, Hardy et al (167, 168) have shown that PPARγ promoter methylation is associated with NAFLD and ALD. This methylation signature can be detected in both genomic liver and in ccfDNA. In this chapter, validation of PPARγ promoter hypermethylation as a liver specific fibrosis signature will be interrogated. Its validation in a HBV, NAFLD and systemic sclerosis cohort was tested.

In his doctoral thesis, Dr. Timothy Hardy produced the first complete methylome map of circulating plasma ccfDNA in NAFLD patients(563). The methylome map generated shows that plasma cell-free DNA harbours exciting, novel methylation signatures that could be used to non-invasively distinguish patients with advanced NAFLD fibrosis. The second part of this study involves validation of these signatures to determine if they can be used to identify patients with severe fibrosis.

6.1.4. Study Aims and Objectives

This is a 'proof of concept' study to develop novel epigenetic biomarkers in NAFLD fibrosis

Step one involves the investigation and validation of PPARγ as a non-invasive generic liver fibrosis biomarker

Step two involves the validation of targets derived from NGS data generated by Dr. Timothy Hardy in his doctoral thesis to establish the potential clinical utility of differential DNA methylation in DMRs in ccfDNA as non-invasive fibrosis biomarkers.

6.2. MATERIALS AND METHODS

Plasma DNA methylation as a biomarker for stratification of mild and severe liver fibrosis in non-alcoholic fatty liver disease

6.2.2. Experimental overview

6.2.2.1. Establishment of PPARγ as a generic liquid fibrosis biomarker

Differential CpG methylation densities at the human PPARγ promoter have been quantified in circulating cell-free DNA (ccfDNA) and shown to correlate with cirrhosis progression in metabolic liver disease (ALD and NAFLD)(168) (564). Clinical utility of a PPARγ fibrosis signature will be validated in a NAFLD, HBV cirrhotic and a systemic sclerosis (SSc) cohort.

6.2.2.2. Development of a NAFLD specific liquid fibrosis biomarker

The global 'Precision Medicine Initiative', co-ordinated by the National Institutes of Health (NIH) and multiple other research centres, promotes a benchmark for clinical and basic science researchers to understand how a person's genetic profile, environment and lifestyle (epigenetic profile) can be developed to guide disease management. In recognition of this universal health objective, a previously generated methylome map of circulating cell free DNA derived from 26 histologically defined NAFLD patients (14 mild fibrosis, 12 severe fibrosis) was consulted (Hardy et al). Bioinformatic analysis identified > 750 differentially methylated regions (DMRs) and over 16,000 differentially methylated loci (DML). Such epigenetic markers represent potential novel methylation signatures that will be investigated as novel biomarkers to non-invasively distinguish patients with advanced NAFLD fibrosis.

6.2.2.2.1. Selection of candidate DMRs from plasma cell-free DNA in NAFLD patients

The first complete methylome map of circulating plasma DNA in NAFLD patients was completed by Dr. Timothy Hardy from ccfDNA from NAFLD patient plasma using the Illumina HiSeq 2500 platform (558). Analysis of data and preliminary alignment was performed with Bismark™. DMR/DML testing was performed using BS-Seq™

6.2.2.2.2. Primer construction

Design The analysis yielded >750 differentially methylated regions (DMRs) and over 16,000 differentially methylated loci (DML). Further parameters were introduced (> 20% methylation change in identified DMRs) reducing the pool to 20 DMRs. Target sequences were identified as coding for 4 DMRs that were hypo (2) and hyper-methylated (2) in severe liver fibrosis. Sequences of interest were characterised using the genome browser Ensembl ™ http:// www.ensembl.org /Homosapiens /Info /Index and were found not be associated with specific genes. PyroMark Assay Design version 2.0 software was used to design primers for the DMR of interest. Primer set assays are designed and scored out of 100. These scores (ideally >70) are calculated based on numerous primer parameters, e.g. mispriming potential, the primer length and propensity for primer dimer. For optimal performance during PCR reactions, primers which bind over non-CpG cytosine bases are preferred and keep the PCR product size short (bisulfite converted DNA is highly fragmented; small PCR fragments (up to 300 base pairs) are optimal for successful amplification of region of interest.) ***See table 7.1***

Table 6.1. A-H Primer Design summary

DMR Number 1

Primer Set A			**Score: 71 Quality: Medium**		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	MZ1.1-F	TTATGTGAATTTAGGAAGTAGAGG	24	62.7	33.3
PCR	MZ1.1-R	AAACCATTAACTCCAAAAAAAAAT	24	65.3	20.8
Sequencing	MZ1.1-S	AAACTAAAAAACAATAATAC	20	42.9	15.0
Target Polymorphisms	Position2				
Sequence to Analyze	A/GATCTCA/ GACTCACAAC CACCTCTACT				

DMR Number 3

Primer Set B			**Score: 75 Quality: Medium**		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	MZ3.1-F	AGTAATTTAGAGTTTGGGAGTTAG	24	61.3	33.3
PCR	MZ3.1-R1	TCAACAATCCTAACCTTTCTCTAT	24	64.3	33.3
Sequencing	MZ3.1-S1	TCTCTATAAATCCTAAAAAC	20	45.1	25.0
Target Polymorphisms	Position3				
Sequence to Analyze	TCA/GCTAAC TCCCAAACTC TAAATTACTT				

Primer Set C			Score: 74 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	MB3.3F1	GAGAGTAGGGTTTTGAGGTAGGAA	24	68.0	45.8
PCR	MB3.3R1	TACCTCCCCATCCCTCTACC	20	69.0	60.0
Sequencing	MB3.3S1	GGTAGGAAATGGAGTAGAGA	20	52.8	45.0
Target Polymorphisms	Position11				
Sequence to Analyze	GAGC/TGAGA GGTAGGGTAG AGGGATGGGG A				

DMR Number 5

Primer Set D			Score: 75		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	MZ5.4-F	TGTTATTATTGGTTTTGGAAGAAA	24	65.7	25.0
PCR	MZ5.4-R	CCACAATACCCAACCTAATTATCT	24	66.3	37.5
Sequencing	MZ5.4-S	TTAAATAATAACCCTAACAC	20	45.9	25.0
Target Polymorphisms	Position7				
Sequence to Analyze	A/GCTTTTCC AATTCCAATA ACTTTTAA				

DMR Number 6

Primer Set E			Score: 79 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	MZ6.1-F	AAATTAGTTGAGTGTGGTGGTATA	24	63.5	33.3
PCR	MZ6.1-R	ACAAACCCAACATTCTTTAATTTA	24	64.9	25.0
Sequencing	MZ6.1-S	TAGTTTTAGTTATTTGGAAG	20	46.2	25.0
Target Polymorphisms	Position5, Position6				
Sequence to Analyze	GTC/TGAGAT GGGAGAATTG TTAGAGTTTA GGAGGTC/TG AGGTTGTAGT GAGTTGAAAT				

Primer Set F			Score: 81 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	MZ6.3-F	ATTTGTGTTGTGGAAAGGTTTATT	24	66.8	29.2
PCR	MZ6.3-R	CAAAATCTCACTACAACCAAATTT	24	65.4	29.2
Sequencing	MZ6.3-S	CTCAAAAAAAATTCATTTTA	20	49.5	15.0
Target Polymorphisms	Position9				
Sequence to Analyze	CA/GCACTAA AAACACACAA ATACAAAAA				

Primer Set G			Score: 80 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	MB6.1F1	GGTTTGATGGTTAGATGGGTATG	23	68.3	43.5
PCR	MB6.1R1	AAAAAACAAACCTACCCCTTTTC	23	67.6	34.8
Sequencing	MB6.1S1	TGGTTAGATGGGTATGAAG	19	52.7	42.1
Target Polymorphisms	Position4				
Sequence to Analyze	GTTC/TGTGG ATATGGATGT AGAGTTTTTT G				

Primer Set H			Score: 72 Quality: Medium		
Primer	**Id**	**Sequence**	**Nt**	**Tm, °C**	**%GC**
PCR	MB6.3F1	TTTGAGAATTAGGAAAGTTGATGG	24	67.5	33.3
PCR	MB6.3R1	AAATAAACCTCATCCAATCCATTA	24	66.7	29.2
Sequencing	MB6.3S1	AACTAAAACATAAATCTTCT	20	44.5	20.0
Target Polymorphisms	Position18, Position19				
Sequence to Analyze	CTCA/GCCTT TAAAATCAAA CA/GTAAACT ATAACTTATA CCATCAACT				

Validation Primers were optimized in terms of concentration, annealing temperature (50°C versus 55°C versus 60 °C) and reaction components Magnesium chloride($MgCl_2$) and Q-solution (Qiagen). PCR primer products were visualised by agarose gel electrophoresis.

6.2.3. Study cohorts

Cohorts to validate a PPARγ fibrosis signature

6.2.3.1. Scleroderma Cohort

Systemic sclerosis (SSc) patients (n=30) were recruited from a study site managed by Professor Jörg Distler, Professor for translational matrix biology, University of Erlangen-Nuremberg. Blood samples were collected subject to patients' written consent. Recruited patients fulfilled the American College of Rheumatology (ACR)/ European League against Rheumatology (EULAR) criteria for the diagnosis of systemic sclerosis. SSc was classified according to the conventional criteria defined by LeRoy et al (565). "Diffuse SSc" was diagnosed if the skin thickening extends proximal to the elbows and knees or includes the trunk, while "Limited SSc" was diagnosed if the skin thickening was confined to the elbows and knees, or to the face. Thirty SSc patients were recruited in total; 18 had limited cutaneous SSc and 12 had diffuse cutaneous SSc. Information collected at time of blood sample collection involved clinical details (gender, age, weight, height, disease duration,

organ involvement) and laboratory data (including scleroderma related antibodies). Lung involvement was considered present if there was evidence of pulmonary fibrosis or pulmonary arterial hypertension. Heart involvement was defined by a past/current diagnosis of congestive heart failure, cardiac arrhythmia, pericarditis, a pericardial effusion, or cardiomegaly.

6.2.3.2. NAFLD and Hepatitis B virus cirrhotic cohorts

HBV cohort (n=13); patients were positive for hepatitis B surface antigen. NAFLD patients were diagnosed as previous. Clinico-demographic were recorded as described in section 2.2.

Cohort to validate novel DMR fibrosis biomarkers

6.2.3.3. NAFLD Index cohort

ccfDNA was extracted by Hardy et al. from 26 patients with biopsy confirmed NAFLD (Freeman Hospital, Newcastle Upon Tyne, UK). Inclusion and exclusion criteria for the diagnosis of NAFLD and recording of clinico-demographic details were as described in chapter 3 and were collected at the time of liver biopsy. Clinical characteristics of this cohort are reported in the results section **(*table 6.3)***

6.2.4. Ethics

For the Turkish NAFLD and Hepatitis B cohorts, use of human tissue was approved by Koç University Ethics Committee for Clinical Research (18.9.2015- 2015.215.IRB1.020). For the Newcastle NAFLD cohorts, Ethics were approved by Newcastle and North Tyneside Local Research Ethics Committee (approval number H10/H0906/41). For the scleroderma cohort, use of human tissue was approved by University of Erlangen-Nuremberg Ethics Committee for Clinical Research.

6.2.5. Procurement/storage of plasma samples from human blood

Whole blood was collected into EDTA tubes and the plasma was separated by centrifugation for 10 min at 3000rpm followed by transfer to new tubes and re- centrifugation for a further 10 min at 3000 rpm. The plasma was aliquoted and stored at -80 °C

6.2.6. ccfDNA extraction

Ccf DNA was extracted from plasma using QIAamp DNA Blood Mini kit (Qiagen, Germany, catalogue no: 51106). Plasma was lysed at 56°C for 10 minutes. The lysate was processed and transferred to spin columns as per manufacturer's instructions. When the sample processing was complete, cell free DNA was eluted in 20ul nuclease free water and stored at -80°C.

6.2.7. Bisulfite modification

EZ DNA Methylation Gold TM Kit (Zymo Research, Irvine, CA, USA) was used for bisulfite conversion of genomic DNA. Cell free circulating and liver tissue DNA were bisulfite modified by incubating at 98°C for 10 min and 64°C for 2 h and 30 min. Product was transferred into columns; desulphonated and washed according to manufacturer's protocol, eluted in elution buffer (10 µl) and stored at -20 degrees until required for use. A 5µl of bisulphite modified cell free DNA was amplified in a PCR mix containing 2µl of forward and reverse primer, 12.5µl of HotStarTaq Master Mix Kit (Qiagen, Germany, catalogue no: 203445) or Pyromark PCR kit (Qiagen, Germany, catalogue no: 978703) and 5.5µl of water. 2.5µl Q solution and 1.5µl $MgCl_2$ (25mM/ml) were added. Amplification of DNA was performed in a thermocycler according to the following PCR conditions: one cycle at 95°C for 6 min, followed by 50 cycles of 95°C for 30 s, annealing temperature of 55°C for 30 s and 72°C for 30 s, followed by one cycle at 72°C for 30 s.

6.2.8. Pyrosequencing analysis

Methylation of specific cytosines within CpG dinucleotides was quantified by pyrosequencing using a Pyromark Q96 ID (Qiagen) instrument. PCR and sequencing primers were obtained from a custom designed assay for PPARγ (forward primer GGAAAGAGGGGTTTTAAGTTTAGG; reverse primer CAATAACCTTTTCTTTTCCTACC; sequencing primer GGGGTTTTAAGTTTAGGAG) and assays designed for specific DMRs as described previously (EurofinGenomics, Germany.). 10µl of biotin-labelled PCR product was used in each well and combined with streptavidin- coated sepharose beads, washed in 70% ethanol, denatured in denaturation buffer (Qiagen, PyroMark Denaturation Buffer, 979007) and washed in a wash buffer (Qiagen, PyroMark Wash Buffer, 979008). Sequencing primers

were annealed to DNA product at 80°C. The samples were analysed in duplicate, and the mean of the two measurements was used as the final value. Assay efficiency was validated by fully unmethylated as well as fully methylated DNA (Qiagen, EpiTect PCR Control DNA Set, 59695). CpG methylation data was analysed by Pyro Q-CpG software 1.0.6.

6.2.9. Statistical analysis

Continuous normally distributed variables were represented as mean ± SD. Categorical and non-normal variables were summarised as median and range. χ^2 test or Fisher's exact test was used to determine the distribution of categorical variables between groups. To compare the means of normally distributed variables between groups, the Student's t test was performed. To determine differences between groups for continuous non-normally distributed variables, medians were compared using the Mann–Whitney U test. Statistical analyses were performed using SPSS software version 24.0 (SPSS Inc, Chicago, USA) and GraphPad Prism Software.

6.3. RESULTS

Differential PPARγ promoter methylation as a biomarker for advanced liver fibrosis

Exploration beyond metabolic liver disease

6.3.1. Patient clinico-demographic details

The clinico-demographic details of the study cohorts are shown in ***table 6.2 and table 6.3.***

For the control cohort (n=22), subjects were age-matched and had no signs or symptoms of liver disease, and no history of chronic illnesses***.***

Table 6.2. Characteristics NAFLD and ALD cohort

	Age (years)	Gender % male	BMI (kg/m2)	Diabetes (%)	ALT (IU/L)	AST (IU/L)
NAFLD cirrhotic Cohort N=13	56 +/- 7	10/13 77%	29.8 +/- 3.2	69	33+/-23	54+/-36
Hepatitis B cirrhotic cohort N=13	51 +/- 7	10/13 77%	26.5 +/- 2.4	38	47 +/- 50	80 +/- 64
Data expressed as mean +/- SD, BMI = body mass index						

Table 6.3. Characteristics of the Systemic sclerosis cohort

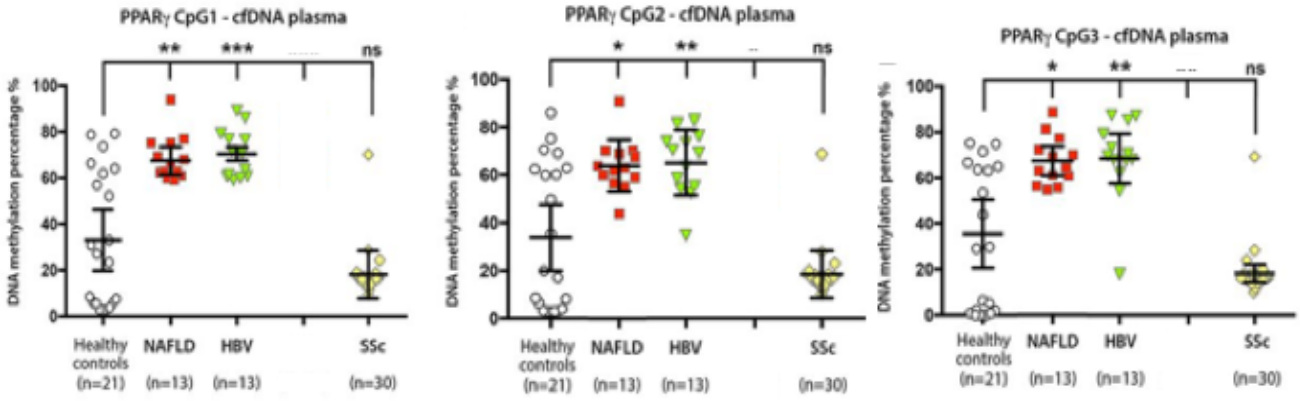
Systemic Sclerosis Cohort (n=30)	
Age (years)	55 +/- 14
Gender (% male)	10/20 (50%)
BMI (kg/m2)	26 +/- 3.8
Diffuse cutaneous limited SSc	12 (40%)
Disease duration (years)	7.5 +/- 4
Heart involvement	2 (7%)
Lung involvement	11 (37%)
DLCO (%)	71.7 +/- 17
Anti-nuclear antibody positive	30 (100%)
Anti-centromere antibody positive	11 (37%)
Anti-topoisomerase I antibody positive	12 (40%)
Data expressed as mean +/- SD, BMI = body mass index	

6.3.2. PPARγ methylation Hep B/NAFLD /Scleroderma cohort

Hypermethylation was detected at all three CpGs in ccfDNA in the cohorts of patients suffering from cirrhosis caused by chronic HBV infection and NAFLD. The levels of hypermethylation resembled that found in patients with cirrhotic NAFLD (CpG1: 86%; CpG2: 65%) and ALD in the original study by Hardy et al (168). Methylation density in the Turkish NAFLD cohort mirrored levels detected in the HBV cohort.

To determine if hypermethylation is specific to fibrosis of liver origin, methylation in a cohort of patients with limited and diffuse systemic sclerosis (SSc) ccfDNA in patients who have various combinations of skin, lung and kidney fibrosis, but no hepatic fibrosis was quantified (566). All three CpG sites in SSc were hypomethylated with similar methylation densities between individual patients with SSc. (CpG1: 16%; CpG2: 9%; CpG3:16%) ***Figure 6.2.*** (567)

***Figure 6.2. PPARγ* methylation Hep B/NAFLD/HCC/Scleroderma cohort**



Plasma cell-free DNA methylation as determined by pyrosequencing at CpG1, CpG2 and CpG3 within the human PPARγ gene promoter from patients with NAFLD, HBV or SSc. n, shows the number of individual patients within each cohort.

DNA methylation was quantitatively measured and expressed as a percentage.

*Error bars represent mean values±95% CI; *P<0.05, **P<0.01, ***P<0.001.*

NAFLD, non-alcoholic fatty liver disease; HBV, Hepatitis B virus; SSc, systemic sclerosis

6.3.3. Patient clinic-demographic details

6.3.3.1. NAFLD Index Cohort

A total of 26 patients with NAFLD were identified who had a liver biopsy to investigate abnormal liver enzymes or to stage fibrosis in those with imaging evidence of steatosis. Fourteen had mild fibrosis (Kleiner 0-2) and 12 had severe (Kleiner 3-4) fibrosis. The demographic and laboratory characteristics of the NAFLD Index cohort of patients are shown in ***table 6.4***.

Table 6.4. Clinico-demographic details NAFLD index cohort

N=22	**Mild NAFLD fibrosis (F0–2)**	**Advanced NAFLD fibrosis (F3–F4)**	**p Value**
	n=14	**n=12**	
Age (years)	57±7	59±12	0.56*
Gender (male)	29%	67%	0.052†
BMI (kg/m2)	36.0±5.5	36.0±7.3	0.996*
Diabetes	50%	67%	0.39†
ALT (IU/L)	55±37	62±19	0.55*
AST (IU/L)	39±13	53±12	0.01*
ALB (g/L)	46±3	45±4	0.37*
Platelets (×109/L)	234±54	223±70	0.67*
AST/ALT ratio	0.80±0.23	0.91±0.27	0.29*
NAFLD fibrosis score	−0.87±0.95	−0.34±1.14	0.22*

Data expressed as mean ± SD or median (range).
*Student's t test.
†χ^2 test.
ALB, albumin; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; NAFLD, non-alcoholic fatty liver disease.

6.3.4. Library of candidate DMRs

20 potential signals (DMRs) were identified for replication from the cell free DNA extracted from the NAFLD index cohort sequenced by TH (563). Preliminary work involved validation and optimisation of 32 primer sets for the 20 available signals. 20 primers were successfully optimised and validated. To refine target selection further, the signals were tested in healthy controls (n=14). 4 signals were appropriately hypo/hypermethylated in 'healthy controls', representative of "mild" disease and were selected for replication in this study ***(table 6.5).*** The signals selected for replication were not associated with specific genes when entered into the UCSC genome browser. Assay efficiency of the 8 selected primers were validated with unmethylated (0% methylated) and methylated DNA (100% methylated) control DNA (Qiagen, EpiTect PCR Control DNA Set, 59695). R^2 were obtained as shown in ***Table 6.6***

Table 6.5. Target sequences

Target Sequence	**Primer Pair**
DMRS Hypermethylated in Severe NAFLD	
Chr3: 12,648,654- 12,648,975	MZ 1.1
Chr2: 85,524,323-85,524,448	MB 3.3, MZ 3.1
DMRS Hypomethylated in Severe NAFLD	
Chr1: 247,827,900-247,828,693	MZ 5.4
Chr20: 28,564,764- 28,565,358	MB 6.1, MZ 6.1, MZ 6.3, MB 6.3

Table 6.6 DMR associated Primer Validation			
Primer Number	Number of CPGS	Number of CPS Validated Pyro	R^2
MZ 1.1	2	CpG1 CpG 2	0.99699 0.99426
MZ 3.1	1	CpG 1	0.86079
MB 3.3	1	CpG1	0.980
MZ 5.4	1	CpG 1	0.95609
MZ 6.1	1	CpG 1	0.99716
MZ 6.3	1	CpG 1	0.98835
MB 6.1	1	CpG 1	0.976
MB 6.3	2	CpG 1	0.9772

6.3.5. Plasma DNA methylation of candidate DMRs

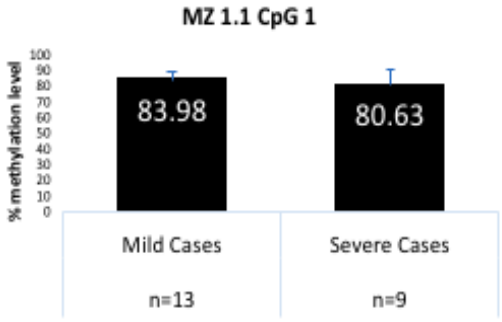
The samples used for validation had been prepared and sequenced previously (TH). The objective was to validate the data from whole genome methylation analysis by performing pyrosequencing on them. CpG methylation density at 4 DMR targets (using 8 primer sets) in 13 cases of mild fibrosis and 9 cases of severe fibrosis was performed. Using tested and optimised primers ***(Table 6.6)*** the amount of DNA methylation at each CpG loci was quantitatively measured in 21 samples ***(Table 6.7, Figure 6.3)*** DNA methylation at CpG loci in patients with severe versus mild fibrosis did not reach statistical significance between the patients with mild and severe NAFLD for any of the 4 target DMRs.

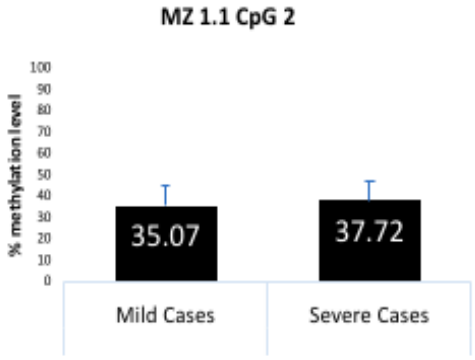
Table 6.7. CpG methylation densities 4 target DMRS, NAFLD Index cohort (n=21).			
	Average CpG methylation (%) **Mild**	Average CpG methylation (%) **severe**	P-Value
Hypermethylated in severe			
DMR 1			
Primer MZ 1.1			
CpG 1	84 +/- 20%	81 +/- 29%	ns
CpG2	35 +/- 32%	38+/-36%	ns
DMR 3			
Primer MZ 3.1	82 +/- 13%	79 +/- 13%	ns
Primer MB 3.3	37+/- 14 %	38 +/- 10%	ns

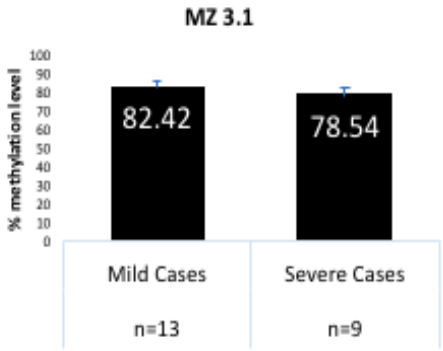
Hypomethylated in severe			
DMR 5			
Primer MZ 5.4	77 +/- 16%	87 +/- 17	Inverse
DMR 6			
Primer MZ 6.1	69 +/- 16%	72 +/- 9%	Inverse
Primer MZ 6.3	87 +/- 11%	86 +/- 9%5	ns
Primer MB 6.1	47 +/- 15%	43 +/- 16	ns
Primer MB 6.3			
CpG 1	86+/- 7%	89 +/- 5%	Inverse
CpG2	73 +/- 8%	79 +/- 5%	Inverse

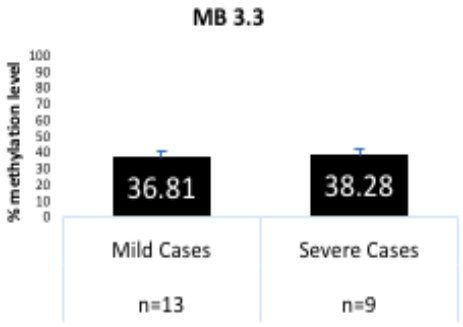
*DNA methylation is quantitatively assessed as mean and SD percentage
**ns= no significant differences between mild and severe fibrosis groups
** Inverse; CpG sites predicted to be hypo/Hypermethylated in severe were found to be hyper/hypo methylated relative to mild fibrotic population; contradicting predictions therefore invalid.

DMR 1 and DMR 3; Hypermethylated in severe fibrosis

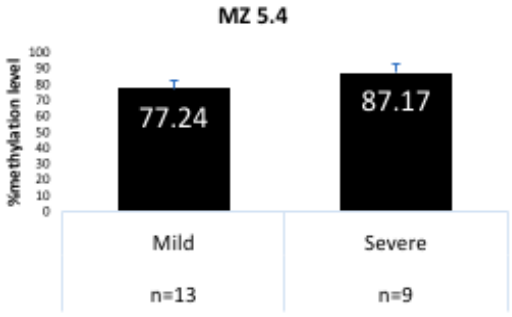


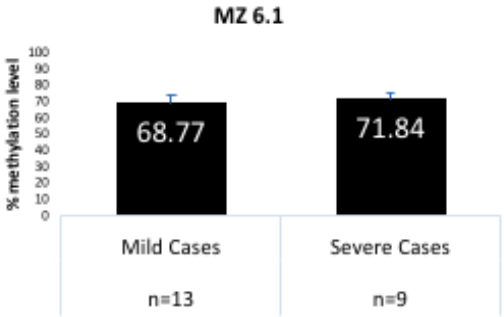


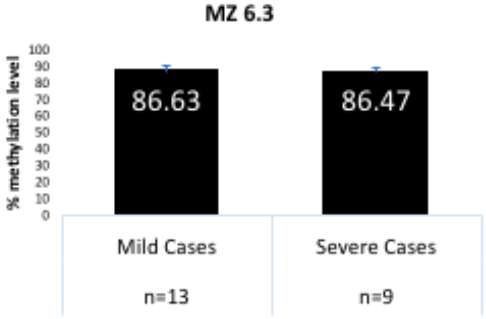


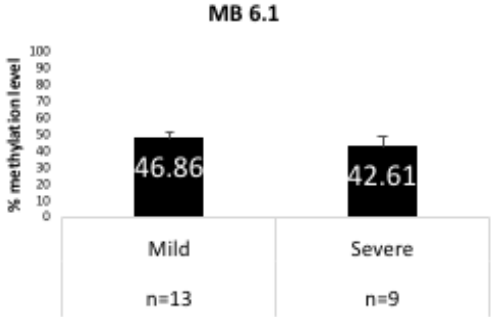


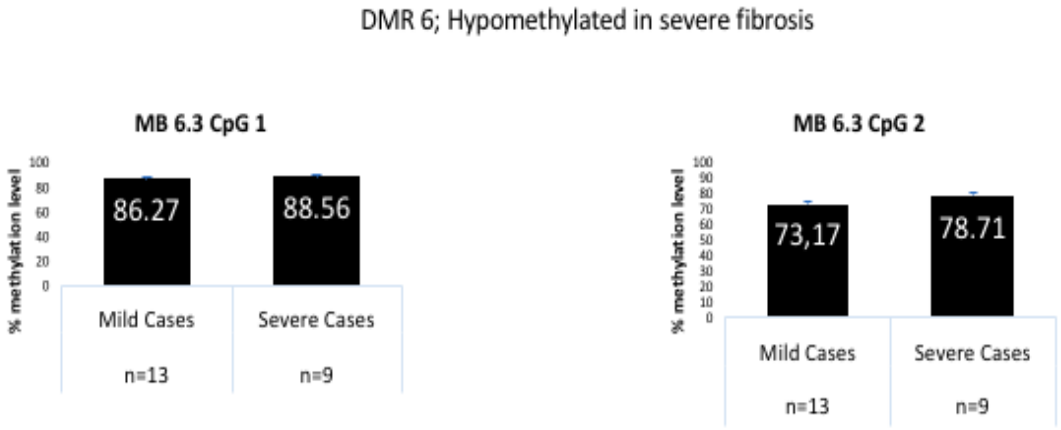
DMR 5 and DMR 6; Hypomethylated in severe fibrosis











DNA methylation is quantitatively measured as expressed as a percentage.

Figure 6.3. Plasma DNA methylation at each CpG in target DMRs

6.4. DISCUSSION

The development of ccfDNA methylation quantification as a diagnostic modality is challenging (219). This body of work investigates both a DNA methylation liver fibrosis biomarker and a NAFLD specific liver fibrosis biomarker.

6.4.1. PPARγ as a liquid fibrosis Biomarker

The original work establishing hypermethylation of PPARγ promoter as a fibrosis biomarker in ccfDNA in ALD and NAFLD was conducted by TH in his thesis (563). This study validated this target in NAFLD, HBV and systemic sclerosis and thus has paved the way for the development of a serum biomarker that monitors fibrosis progression in liver diseases of multiple aetiologies. PPAR γ is classified as an anti-fibrotic gene, induced in NAFLD where the observed hypermethylation in its promoter region would suggest that it is appropriately turned off in advanced NAFLD (568, 569). It has been targeted by insulin sensitising drugs in humans (PPARγ agonists)(570-572) and is associated with improved transaminase levels, steatosis, ballooning, lobular inflammation and fibrosis (573). However, cell free PPARγ as a surrogate liver DNA methylation marker in the blood is challenged due to the high expression of this gene in extra-hepatic tissues and the observation of transient PPARγ promoter methylation in human skeletal muscle in response to aerobic activity and caffeine consumption (218, 574).

6.4.2. The origins of ccfDNA

In disease states, DNA from dying cells is released into the bloodstream (219). NGS does not differentiate the tissue origins of ccfDNA. Various methods to establish the tissue origin of ccfDNA have been explored (575-583), however, bioinformatic deconvolution processes are the most informative and involve the generation of a 'tissue map' of plasma DNA (584, 585). The most up to date paper establishing the contributors to ccfDNA in healthy subjects is by Moss et al using the Illumina Infinium array (219) where ccfDNA contributors included granulocytes (32%), erythrocytes (29%), monocytes (11%) and lymphocytes (12%). Solid tissues of ccfDNA include vascular endothelial cells (9%) and hepatocytes (1.2%). A recent study using 3 targeted hepatocytes markers has also shown that approximately 1% of DNA is derived from hepatocytes, (582) supporting the assumption that ccfDNA from this tissue

type is cleared via the blood. Looking specifically at the origin of ccfDNA in sepsis, it was found that the levels of hepatocyte ccfDNA were strongly correlated with levels of ALT- a marker of hepatocyte damage.

6.4.3. *Problems associated with DMR target validation- ccfDNA*

ccfDNA as a molecular marker for the diagnosis of fibrosis presents a variety of analytical and technical challenges summarised in ***figure 6.9*** which may explain which may have contributed to our inability to validate the selected signals. ***ccfDNA as a substrate:*** ccfDNA is highly fragmented (170-500 bp) and circulates at very low concentrations. Such a composition is not favourable to DNA bisulfonation. A study which evaluated DNA quality derived from FFPE samples (formalin-fixed paraffin-embedded) in 1000 samples on a Qc platform (BioCule ^{TM}) demonstrated tremendous variability in both the amount and types of DNA damage in human sample DNA (586). ccfDNA is more unstable than DNA extracted from FFPE therefore sample variability is highly likely to negatively affect the final results (586). ***Blood sample collection;*** Blood samples obtained for validation contain large amounts of haematopoeic cells. All samples in this study were separated within the 6-hour recommended window to avoid significant cell apoptosis with onsite centrifugation. ***Plasma separation*** all efforts were made to ensure adequate plasma separation. The protocol used included a description clearly defining the interface “buffy coat” to ensure minimal WBC carry over. ***ccfDNA extraction method***; In a study evaluating commercial kits for the purification of circulating cell free DNA, diefenbach et al concluded that the commonly used spin column-based Qiagen QIAamp circulating nucleic acid kit was the most consistently performing kit across two evaluation assays employed (587). The Qc technique employed was Bioanalyzer ^{TM} microfluidic separation and optimal methods were used for quality control and yield quantification.

6.4.4. Problems with DMR Target validation- NGS

The evolution of NGS technologies over the last decade has led to its widespread adoption in both basic and clinical science. Unfortunately, this advancement has occurred in the absence of clear recommendations for validation of NGS bioinformatics pipelines and have contributed to inconsistencies in clinical laboratory practice. Cognisant of this problem, the

Association of Molecular Pathology (AMP), with organizational representation from the College of American Pathologists and the American Medical Informatics Association, have developed best practice consensus standards and guidelines for the validation of clinical NGS bioinformatics pipelines (230). ***Reproducibility crisis and workflow decay;*** NGS is now available at a lower cost than traditional Sanger sequencing and is now widely employed (588). However, reproducibility of results is frequently not tested in the bioinformatic field resulting in a phenomenon called the "reproducibility crisis"(589). Large scale genomics data demand complex computational workflow environments; and data validation necessitates duplication of the software environment (operating systems, base software dependencies and configuration settings under which the original analysis was conducted) (590). This is complicated by a phenomenon called "workflow decay" (591), which is a collective term for the problems generated by the evolution of the technological environment and the redundancy of third party web resources. Failure to validate DMR targets in this study may necessitate looking back on the consistency of reporting in the original sequencing data. ***Inherent error rates in NGS*** NGS data has shorter read lengths with consequent higher error rates in comparison to traditional Sanger sequencing (592-595). A comparative study of k-spectrum-based error correction methods for next- generation sequencing data analysis demonstrated that factors such as coverage depth, read length and genome size all serve to influence the performance of individual k-spectrum-based error correction methods. It is important to select the most appropriate methods for error correction specific NGS datasets to improve the accuracy of the results (596). ***Data analysis problems***. A major contributing factor stems from the enormity of data generated in a sequencing run. For example, a typical BAM file for a single 30X human whole-genome sample is approx. 90 GB (586). This generates issues with regard to data storage. The next problem relates to the choice of analysis tools on offer. There are over 3000 analysis tools listed at OMICtools (a directory operated by OMICX). The final challenge, relates to clinical interpretation. For clinical samples, there is a problem with regard to the delivery of a consistent, reliable, interpretation of the sequencing variants. A whole genome sample can have up to 3 million variants(586). To help guide clinicians, the American College of Medical Genetics and Genomics, the Association for Molecular Pathology, and College of American Pathologists

have created a system for classifying variants. However, even with such systems, participating laboratories have seen agreement in only about 34% of cases (586).

6.4.5. Study strength and limitations

Liver biopsy as reference standard the reference standard in this biomarker study is liver biopsy, which is subject to sampling error and inter-observer variability among pathologists as previously discussed. However, DNA methylation status appears to be uniform within the liver implying that genomic DNA methylation signals derived from hepatocytes should not be subject to sampling error. In a study by Zeybel et al, a number of samples were taken spanning the entire area of the liver. CpG methylation status of a number of pro and anti-fibrotic genes demonstrated little intra-individual variation in methylation for each CpG site independent of anatomical location, age and gender (167) .

Candidate genes selection PPARγ expression is not specific to hepatocytes, it will be necessary to confirm that differential methylation detected in the promoter region of PPAR γ is a direct consequence of hepatocyte cell death and that the ccfDNA released is a result of NASH-associated significant fibrosis. At first instance, this could be confirmed by including a marker of cell death such as caspase generated cytokeratin-18 fragments (CK18) which has previously been shown to correlate with histological NASH (597).

Failure to show relationship between target DMRs and ccfDNA in plasma Although disappointing, this study has provided important insights into design principles for effective ccfDNA extraction, primer design and DNA methylation quantification.

6.4.6. Future directions

Further validation studies Epigenetic biomarkers have potential as fibrosis biomarkers. Given the urgency for such a development, there is a drive to promptly translate biomarkers from bench to bedside. However, rigorous steps must include exhaustive validation in different clinical cohorts before they become clinically useful.

Alternatives to GWBS other more cost effective methods could be considered which have a more targeted approach. Avoidance of the harsh BS conversion may also be beneficial. With the advent of single molecule sequencing approaches that permits the direct interrogation of the genome, the analytical precision of the approach might also improve e.g. Nanopore.

This type of sequencing has already been applied to sequence maternal plasma DNA (598). Work could also be complemented by running transcriptomic approaches alongside DNA methylation. Additional insights into NAFLD pathogenesis may be obtained by investigating the tissue contribution towards the nucleic acid pool via the study of mRNA and miRNA.

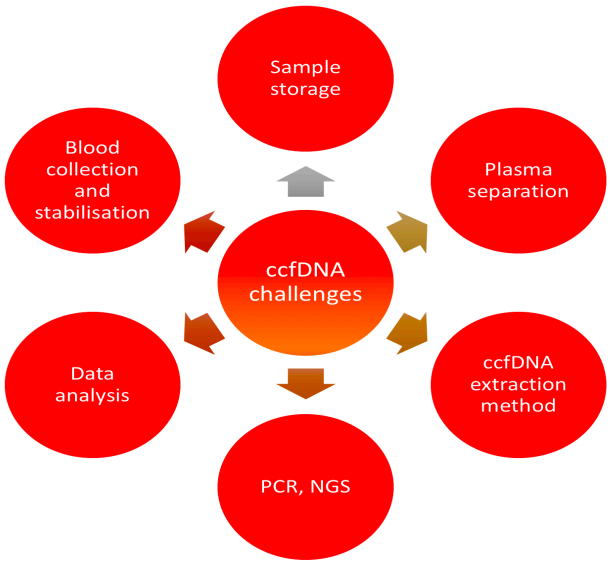


Figure 6.4. Troubleshooting ccfDNA sample acquisition

6.5. CONCLUSION

Further investment is needed to make the translation of DNA methylation signatures from bench to bedside a reality. This study has provided insights into linking genomic information obtained from circulating DNA to its origin anatomy thus bridging the gap between molecular diagnostics and the traditional more organ-based medical practices. Many challenges remain across the NGS workflow, ranging from sample preparation to data analysis. However, new challenges in data analysis are likely to parallel the development of these new technologies and rising to these challenges will be critical to ensure the widespread adoption of this technology to maximise their impact on human health.

CHAPTER 7.

METHYLOME-WIDE SEQUENCING DETECTS METHYLATION SIGNATURES DISTINGUISHING LOW FROM HIGH RISK NAFLD

7.1. INTRODUCTION

A common objective in translational research involves the identification of disease progression signatures. A strategy applied in genomics research to date has involved clustering patients based on gene expression data and coupling a defined gene set with a prognosis if it happens to associate with a statistically significant outcome. However, the validity of this process is challenged in that "random" gene sets may spuriously cluster patients into prognostically variable subgroups and in NAFLD to date such whole exome sequencing studies of genetic variation in liver biopsy have failed to identify significant gene trends (599). In this study, the focus is shifted to the more dynamic epigenome, with the objective to define a 'baseline' risk signature derived from a patient population that has been rigidly stratified according to their phenotype and a "purified" liver biopsy platform consisting almost exclusively of hepatocytes.

7 1.1. Epigenetic regulation of fibrosis progression

A hallmark of liver fibrosis is hepatic stellate cells transdifferentiation to myofibroblasts which is subject to epigenetic regulation (213, 215, 600). DNA methylation is the most extensively characterised epigenetic mechanism where CpG methylation in gene promoters typically represses gene expression however, it has also been observed to be increased in actively transcribed genes if located within intragenic regions (601). The significance of CpG methylation in regulating gene expression has proved highly variable and indeed only ~ 20% of CpGs are dynamically regulated (602). Pertaining specifically to NAFLD, differential DNA methylation was quantified in 69 247 CpG sites in liver biopsies stratified into mild and severe fibrosis. The majority of DMRs became hypomethylated with disease progression (76%), whereas 24% underwent hypermethylation. Subsequent pathway analysis exercises confirmed that repair genes were hypomethylated therefore actively transcribed in advanced NAFLD supporting the hypothesis that methylome-transcriptome interactions that occur in advanced NAFLD can modulate NAFLD outcomes (216). Other work has addressed the potential genetic and epigenetic cross-talk in NAFLD. As an example, the rs738409 Il148Met PNPLA3 SNP (603) may couple with a DMR in the PNPLA3 promoter which is found to be hypermethylated in severe fibrosis (562). Zeybel et al have also reported that differences in methylation at genes implicated in fibrogenesis can stratify patients into

stable mild fibrosis vs severe fibrosis (167). In particular, as validated in the previous chapter; hypomethylation at the PPARγ promoter identifies stable NAFLD and remarkably this epigenetic biomarker can be detected in circulating cell-free DNA raising the potential of a minimal-invasive diagnostic test for fibrosis.

7.1.2. Value of Differentially methylated regions (DMR) analysis across biological samples

The immense complexity in CpG methylation analysis often produces weak, non-specific correlations between CpG methylation and gene expression data limiting the derivation of robust conclusions. While still considering individual CpG sites, practices have evolved whereby the genome is now scanned for clusters of differentially methylated CpG sites spanning a short region i.e. differentially methylated regions (DMRs). DMRs have been instrumental for tissue characterization with broad applications in developmental and aging studies (604-606) and have been characterised as diagnostic and monitoring biomarkers in oncology (607). In this study, using a WGBS technique, novel DMRs between 2 phenotypically distinct types of NAFLD patient (low and high risk) will be characterised. An advantage of this method over array-based methods is that it extracts methylation information directly from the converted DNA sequence rather than read counts. Direct genome sequencing also provides more complete and unbiased genomic coverage with higher accuracy, even in comparison to the most advanced high-density gene arrays such as the Infinium 450 Bead chip array (Illumina), which detects only 1.5% of CpGs in the human genome (608). The overarching goal of this work is to generate and prioritize hypotheses to undergo further investigation.

7.1.3. Laser Capture microdissection reveals cell specific DNA methylation signatures

Studies in human liver tissue have observed loci-specific differential DNA methylation in genes that modulate fibrosis progression in chronic liver disease (167, 609). However, an acknowledged caveat to these studies is that the DNA methylation analysis was performed on whole liver biopsies. Given the heterogeneous nature of whole liver biopsy tissue it is therefore possible that the observed differential DNA methylation may be reflective of changes in cell types and architecture not specifically associated with the fibrogenic process. However, in a study by Hardy et al, based on the hypothesis that DNA methylation is likely

to be cell specific demonstrated that hypermethylation at the PPARγ promoter of plasma DNA correlated with changes in hepatocellular rather than myofibroblast DNA methylation in NAFLD and ALD cirrhosis. This study confirmed that DNA methylation signatures reflect the molecular pathology associated with fibrotic liver disease (168). Prior to this study, cell separation had not been utilised to complement liver tissue DNA methylome studies. Laser capture microdissection (LCM) is an emerging technology that allows isolation of specific cell types while preserving the tissue microenvironment. DNA methylation is a compelling candidate for involvement in high risk NAFLD due to the documented essential role that it plays in HSC transdifferentiation into pro-fibrogenic myofibroblasts (213, 215, 600). This study will stratify liver biopsies by histology using the Kleiner fibrosis scale and use Laser Capture Microdissection (LCM) to ensure that the molecular signature characterised will be attributable to changes occurring almost exclusively in liver hepatocytes.

7.1.4. Study aims and objectives

To detect DNA methylation changes at baseline that distinguishes high risk from low risk NAFLD, the study cohort will be divided into 2 phenotypically distinct two groups. BS sequencing and bioinformatic analysis will facilitate the identification of differentially methylated regions (DMRs) as potential methylation signatures.

The aim of this study is to;

1. Characterise a methylation signature to differentiate low risk from high risk NAFLD
2. Derive a hypothesis relating to disease pathogenesis in low and high NAFLD
3. Consider potential links to novel pathways that control biological processes underpinning low and high risk disease

The data provided by this proof of concept study will provide insights into disease risk in NAFLD, with the intention that future work will address the function of specific loci and the possibility of a DNA-methylation-based diagnostic test distinguishing those with progressive disease from those with stable disease or regression early in the disease process.

7.2. MATERIALS AND METHODS

Methylome-wide sequencing detects methylation signatures distinguishing low from high risk NAFLD

7.2.1. Establishing a platform to characterise an epigenetic signature representative of rates of hepatic fibrosis progression in NAFLD

The Freeman Hospital Liver Unit has an established cohort of 108 well-characterised NAFLD patients that have undergone paired biopsies ***Figure 7.0*** This cohort was stratified into 'high risk/fast progressors' and 'low-risk stable/regressors' groups for analysis. Index biopsies selected for the study had F0-F1 fibrosis. The first biopsy was taken at time of diagnosis with second biopsy taken routinely to monitor disease progression. During a median of 6.6 (range 1.3-22.6) years follow-up, 42% have progressed to advanced Kleiner grade 3-4 fibrosis/cirrhosis, 40% have remained stable and 18% have exhibited disease regression. This resource represents the largest single-centre cohort with patients that have undergone multiple liver biopsies and are still under ongoing clinical follow-up. Phenotyping criteria is listed in ***table 7.0***.

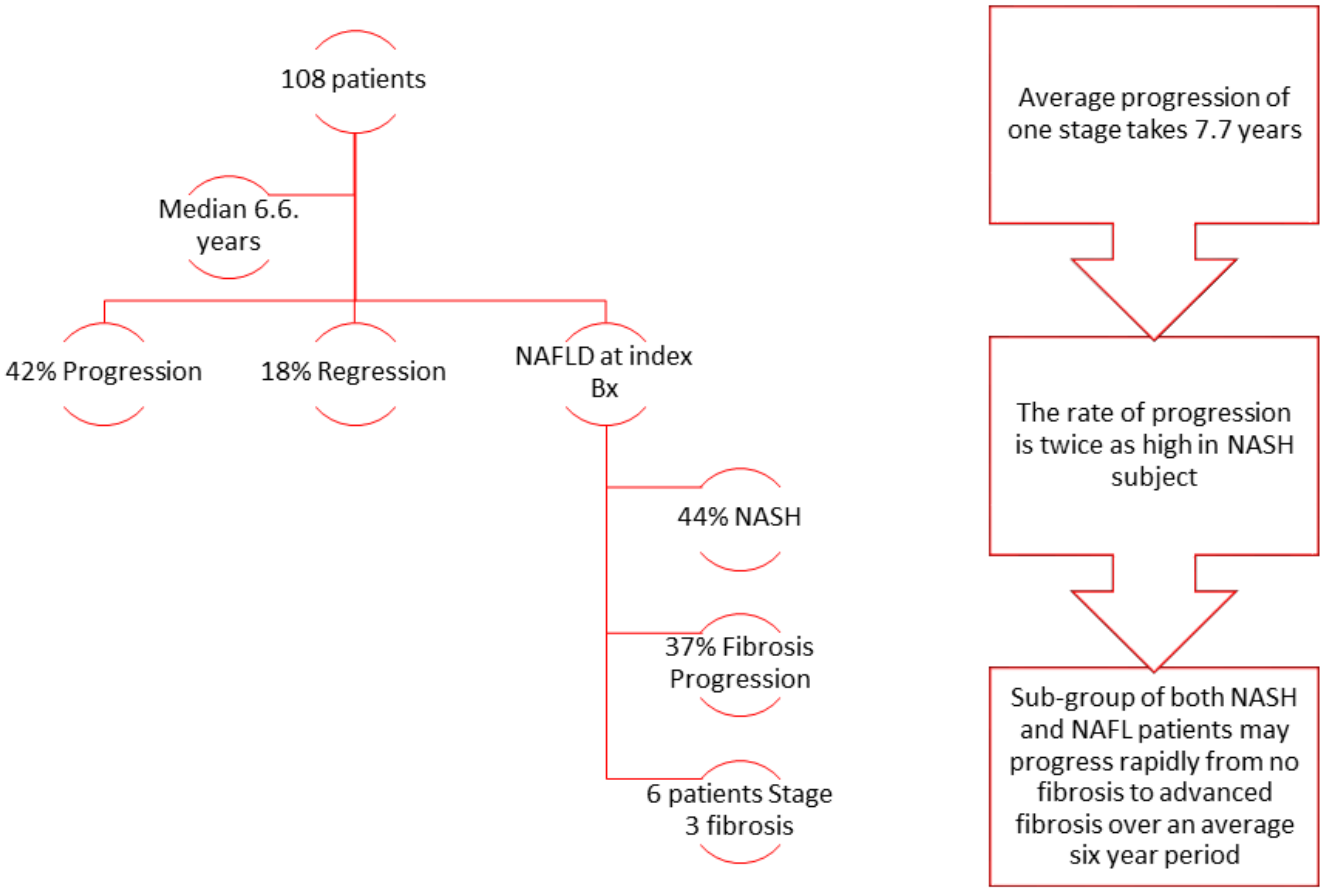


Figure 7.0. UK-DELTA cohort

High risk NAFLD i.e. Fast Progressors (FP)	F0/F1 at index biopsy that has progressed ≥2 stages within 6-7 years
Low risk NAFLD Stable/regressors (SR)	F0/F1 at index biopsy that has remained stable/regressed/progressed at least 1 stage within a period exceeding 8 years

Table 7.0. Phenotypic classification of NAFLD cohort into fast and slow/non progressors

7.2.2. Patient Selection

Patients with 2 or more liver biopsies, taken at least 1-year apart were selected from the UK-DELTA cohort. Index liver biopsies were performed between 1991 and 2011 as part of investigation of abnormal liver function tests, or to stage disease severity, in patients with radiological evidence of NAFLD. Follow-up liver biopsies were conducted between 2001 and 2013 to assess disease progression or as an entry requirement for inclusion in a clinical trial.

Due to the uncertain natural history of NAFLD, local practice is to perform a follow-up liver biopsy at 5-yearly intervals to monitor for disease progression in pre-cirrhotic patients aged <65-years who failed lifestyle intervention. For patients with >2 liver biopsies, the first and last biopsies were used, unless the patient had participated in a therapeutic clinical trial where the pre-trial biopsy was used. NAFLD was diagnosed and clinico-demographic details collected as outlined in section 2.2. Use of human tissue was approved by Newcastle and North Tyneside Local Research Ethics (approval number H10/H0906/41). The information was collected retrospectively from the EPoS NAFLD Registry (http://www.EPoS-nafld.eu/.)

7.2.3. Histological assessment

Percutaneous liver biopsies were performed and NAFLD diagnosed and staged by an expert histopathologists as per NASH CRN criteria outlined in chapter 2, section 2.2.

7.2.4. Preparation of slides for Laser Capture Microdissection (LCM)

Samples on Membrane Slide: FFPE blocks were obtained from the biobank archives of the Molecular Pathology Node Proximity Lab, Newcastle University. The microtome was set to 10 μm and the sections were fixed onto polyethylene naphthalate (PEN) membrane slides (Carl Zeiss Micro Imaging) PEN membranes are highly absorptive in the UV-A range and are simultaneously cut with the sample providing a stabilising backbone. They are certified DNase, RNase and human DNA free (Zeiss Membrane Slide 1.0 PEN NF - Order No. 415190-9081-000)

Staining Slides were hydrated, deparaffinised (Clearene solvent (Leica Biosystems, Germany)), placed in 100% ethanol, dried and then fixed in 70% ethanol for 5 seconds. Slides were stained in hematoxylin for 30 seconds. Excess stain was removed on an absorbent surface. Slides were dipped in Scott's tap water substitute and dehydrated with sequential ethanol dips (ranging 50-100% concentrations) and left to dry in the fume hood for 1 hour.

7.2.5. Laser Capture Microdissection

LCM was performed using the Zeiss PALM Micro Beam; hepatic parenchyma was separated from blood vessels and portal tracts under direct visualisation.

7.2.5.1. Tissue Collection.

Blood vessels and portal tracts were isolated and removed leaving the remaining hepatocytes only. Each segment was between 1-2.5 million µm2 in area and collected in a one Adhesive Cap (Zeiss; AdhesiveCap opaque - Order No. 415190-9201-000*)* in conjunction with PALMROBO (software version 4.6).

7.2.6. Isolation of genomic DNA from LCM Tissue

Genomic DNA and RNA were isolated from LCM Tissue using AllPrep® DNA/RNA FFPE kit (Qiagen, Germany, Cat No: 80234). DNA precipitation was performed by incubating the FFPE liver biopsy in a lysis buffer (Buffer PKD, proteinase K) at 56^{o}C for 15 min then standing on ice for 3 minutes. A DNA pellet was obtained after centrifugation. DNA was purified using QIAamp MinElute spin column (DNA) as per manufacturer's instructions. Samples were stored in the -80^{o}C freezer until further processing.

7.2.7. Generation of sequencing Libraries

The entire volume of genomic DNA from LCM samples was bisulfite modified by incubating with 'lightening conversion reagent' at 98 ^{o}C for 8 minutes, then 54 ^{o}C for 1 hour. The product was transferred into columns; desulphonated, washed with M-buffer and eluted in 10 µl of elution buffer. The total volume of gDNA was mixed with the PrepAmp buffer (5X) and PrepAmp Primer(40uM). In a second tube, PrepAmp buffer, PrepAmp Pre-mix and PrepAmp polymerase were placed on ice. Both tubes were placed in a thermocycler (98^{o}C for 2 min, 8 ^{o}C for 1 min, hold, 8 ^{o}C for 4 minutes, 16 ^{o}C for 1 minute, 22 ^{o}C for 1 minute, 28 ^{o}C for 1 minutes, 36 ^{o}C for 1 minutes, 37 ^{o}C for 8 minutes, cooled to 4 ^{o}C for 2 cycles) During the 4^{o}C step of Cycle 1*,* 5.05 µl PrepAmp mix was added and during Cycle 2, 0.3 µl PrepAmp Polymerase was added*.* DNA purification and concentration was performed by adding a 7:1 ratio of DNA binding buffer to 25ul of product, spun in a collection tube, washed and eluted in DNA elution buffer. Amplification was performed in a reaction mixture containing LibraryAmp Master Mix, LibraryAmp primers and the purified BS converted DNA. The sample was incubated in the thermocycler (94^{o}C for 30 seconds for 1 cycle, the 94 ^{o}C for 30 seconds, 45 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds, 68 ^{o}C for 1 minute

and 68 °C for 5 minutes for 5 cycles then cooled to 4 °C). The PCR product was then purified as before. The product was then amplified with the index primer and LibraryAmp Master Mix (2X) in a thermocycler (94 °C for 1 cycle, then 94 °C for 30 seconds, 58 °C for 30 seconds, 68 °C for 1 min, then a final cycle of 68 °C for 5 minutes, then cooled to 4 °C)

7.2.8. DNA Quality Control in Next-Generation Sequencing; Library Quantification

DNA quality control was performed using the Agilent 2100 Bioanalyzer, utilizing fluorescence detection (monitoring fluorescence between 670 nm and 700 nm). Data was displayed as electropherograms. Sizing and quantitation data was presented in tabular form and was exported to excel. Chips were prepared according to the instructions provided with the DNA 7500 Lab Chip kit (Agilent Technologies GmbH, Waldbronn, Germany). The gel-dye mix was prepared by mixing 400 µl of the gel matrix with 20 µl of the dye concentrate and the mixture was filtered through a spin filter. The separation chip was filled with the gel matrix/dye mixture and 5 µl of the markers was added to each sample well. After adding 12 samples (1 µl each) to the sample wells and the DNA sizing ladder (1 µl) to the assigned ladder well, the chip was vortexed and run on the Agilent 2100 Bioanalyzer.

DNA quantification was performed using Qubit. Samples were diluted to a 5 ng/µl concentration (estimated by the Bioanalyzer results) and quantified using the QuBit dsDNA HS Assay kit and the QuBit 2.0 fluorometer following the manufacturer's instructions (Life Technologies). Prior to measurement, a five-point calibration curve was established using the supplied standard. One-microliter of the 5 ng/µl solutions were diluted 200-fold in Qubit assay dilution buffer and measured on the fluorometer. Samples that fell below the limit of detection were re-quantified using lower dilutions. Concentrations provided by QuBit were used to calculate the molarity of the initial sample, correcting for the dilution factor and converting to molarity using the average size of the library as detected by the Bioanalyzer. Measures were done in duplicates

7.2.9. Bisulfide sequencing using the illumina platform, bioinformatic and gene network analysis

Sequencing was performed on an Illumina HiSeq 2500. Sequencing reads were aligned to the reference human CRCh38 genome from the ensembl database using Bismark. Data was

smoothed and base calling/testing was done using BSmooth from Bioconductor. *Detailed analysis steps are described in chapter 8.*

The DAVID Gene Functional Classification Tool (http://david.abcc.ncifcrf.gov) was used to group functionally related genes into a number of biological modules for efficient interpretation of gene lists in a network context. This platform uses an algorithm to condense a list of genes into groups of related genes or biology, called biological modules, by sequential mining the complex biological co-occurrences found in multiple sources of functional annotation (610).

7.3. RESULTS

7.3.1. Baseline Characteristics of the UK-DELTA pilot cohort selected for study

This was a retrospective pilot study. 8 patients with paired biopsies were selected from the UK-DELTA cohort for inclusion. The patients were phenotyped into a specific group based on disease progression patterns i.e. low risk or high risk NAFLD***. (Table 7.1)*** The patients were similar in terms of demographic, clinical, biochemical and metabolic profiles. All patients had F0/F1 fibrosis on index biopsy ***(Table 7.2)***

Table 7.1. Patient stratification into high risk and low risk NAFLD

Stage Index Biopsy	*Stage end Biopsy*	*Time Interval*	*Number Stages Changed*	*Phenotype*	*Risk*
1(b)	4	72 months	3	Progressor	High
1(a)	3	84 months	2	Progressor	High
1(b)	4	48 months	3	Progressor	High
1 (b)	3	120 months	2	Progressor	High
0	0	24 months	0	Stable	Low
1 (a)	1	144 months	0	Stable	Low
1 (a)	1	48 months	0	Stable	Low
1 (a)	0	96 months	1	Regressor	Low

Table 7.2. Demographics, laboratory and histological investigations of study population (n=8)				
	Total cohort N=8	**High Risk N=4**	**Low risk N=4**	**P-value**
Age (Years)	53+/-6 (44-59)	52+/- 6	53+/6	0.867
Male, n, (%)	4 (50%)	2 (50%)	2 (50%)	1.000
Diabetes, n, (%)	6 (75%)	3 (75%)	3 (75%)	1.000
Weight (Kg)	90 +/- 15	87+/ 19	93+/10	0.583
Body mass index	34 +/- 4	34+/ 6	35+/3	0.700
ALT (IU/L)	94 +/-66	80+/29	109+/94	0.574
AST (IU/L)	65 +/-49	57+/13	69+/68	0.823
ALP (IU/L)	94 +/-25	89+/34	102 +/ 11	0.556
Albumin (g/L)	46 +/-2	47+/2	44+/ 2	0.06
Platelets ($x10^9$/L)	259 +/-49	254+/73	264 +/16	0.797
HBA1c	6+/-0.8	6+/1	6 +/0.5	0.411
Ferritin (ug/L)	154 +/-134	91+/66	239 +/ 168	0.161
AST: ALT (ratio)	0.66+/-0.17	0.71+/0.22	0.62 +/ 0.15	0.557
FIB4	1.3 +/-0.54	1.34+/0.61	1.22 +/ 0.59	0.803
NAFLD Fibrosis Score	-1.48 +/-1	-1.70+/1.3	-1.32 +/ 0.90	0.658
Steatosis (0/1/2/3)	(0/2/5/1)	0/0/3/1	0/2/2/0	0.202
Ballooning (0/1/2)	(2/6/0/0)	1/3/0	1/3/0	1.000
Lobular Inflammation (0/1/2/3)	(1/6/1/0)	1/2/1	0/4/0/0	0.264
Fibrosis Stage (0/1/2/3/4)	(1/7/0/0)	0/4/0/0	1/3/0/0	0.285
NAS	4+/-4 (2-6)	4+/2	3+/ 1	0.414
NASH (NAS≥4)	6(75%)	3 (75%)	3 (75%	1.000
PNPLA3 ***G Allele Positive***	2 (25%)	1(25%)	1 (25%)	1.000
***Statistical Tests**: student t-test/X^2 **ALT=alanine aminotransferase; AST=aspartate transaminase; ALP=Alkaline Phosphatase; HbA1c= Haemoglobin A1c; NAS= NAFLD Activity Score				

7.3.2. Whole Genome Sequencing of liver parenchymal genomic DNA

The size distribution of genomic DNA was centred on 313bp (range 271-371bp). Genomic DNA was extracted from liver biopsy specimens subject to LCM from the 2 NAFLD groups and used to construct sequencing libraries. Amplified libraries exhibited a mean peak of approximately 300bp containing the sequencing adapters. A representative size distribution profile for the library is shown in ***figure 7.1***

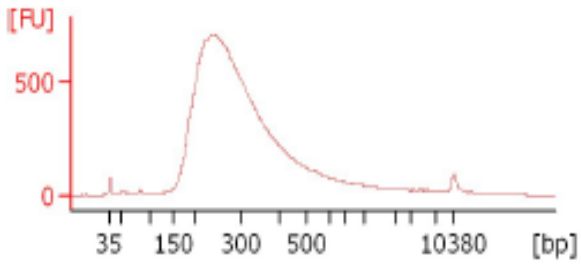


Figure 7.1 Representative Bioanalyzer profile of genomic DNA and WGBS sequencing library.

The liver biopsy tissue DNA in each group underwent NGS using the Illumina HiSeq 2500 platform. The raw sequenced reads were mapped to the human reference genome (Human hg38) in order to investigate novel methylation signatures associated with high and low risk NAFLD.WGBS quantifies DNA methylation at essentially every nucleotide in the genome (602, 611). From a statistical perspective, examining single nucleotide DNA methylation variation would generate inestimable hypotheses in the range of 60 million.

It has been documented that regions exhibiting differential DNA methylation are more valuable in large-scale BS-seq data analysis (611). Consequently, differentially methylated loci (DMLs) *will not* be described in this thesis as the volume of CpGs detected at this resolution was too large and non-specific to derive functional or prognostic significance (612)

7.3.3. Differentially Methylated Regions (DMRs) in NAFLD groups

Regions of the DNA methylome that were significantly differentially methylated between the two NAFLD groups were characterised. Data was smoothed and base calling/testing was done using *BSmooth* from *Bioconductor*. In this analysis, a DMR was defined as an area that contained (a) more than 1000 base pairs (bps) and (b) $\geq$ 70 DMLs. A quartile-based cut-off selection process was employed on the platform and a direct comparison of DMRs between the 2 groups was found to yield 657 novel DMRs with 3578 CpGs. 367 hypermethylated and 289 hypomethylated regions were identified that differentiated the 2 groups. A heat-map of high risk versus low risk disease using methylation in NAFLD specific liver DMRs is shown in ***figure 7.2.*** Percent methylation for each sample relative to the mean methylation at each DMR is plotted.

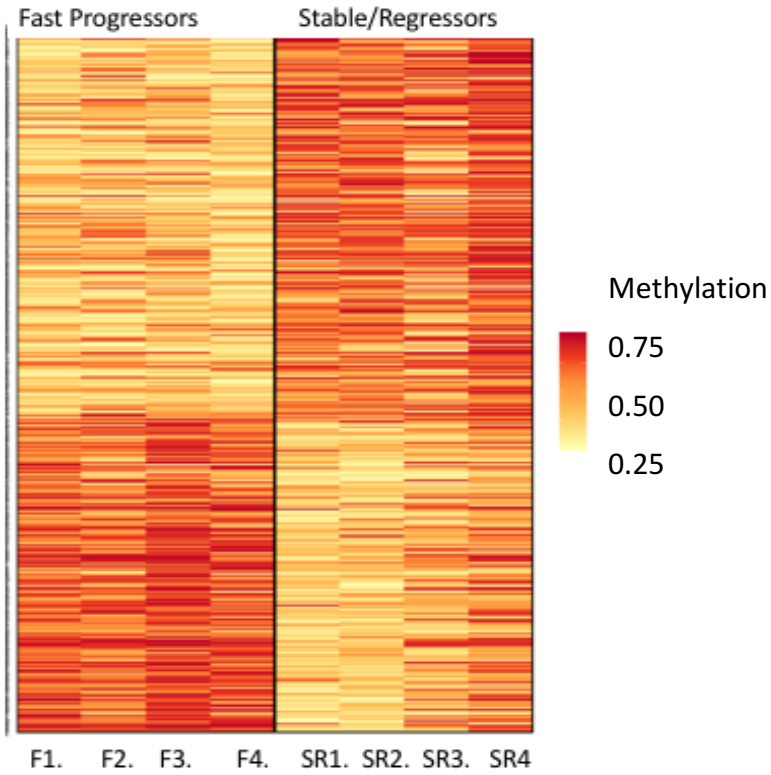


Figure 7.2 Heat-map of fast progressors versus stable/regressors using methylation in NAFLD specific liver parenchymal DMRs

When the DMRs were filtered to exclude DMR reads that represented non-classified functional regions the list was reduced to 268 hypermethylated DMRs, 207 hypomethylated and 22 mixed DMRs ***figure 7.3***

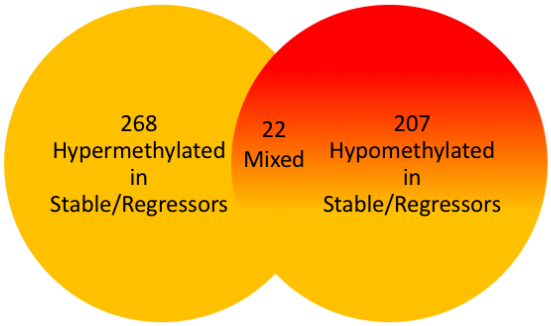


Figure 7.3 DMRs differentiating low risk from high risk disease and those that did not were identified.

The statistical method used to detect differential methylation in this study was BSmooth (compatible with WGBS data). This platform involves a smoothing step (polynomial logistic regression with tri-cube weight- using methylation measures from neighbouring sites) to calculate a t test-like test statistic (t-statistic) based on locally estimated variance for CpG modelling and testing. DMRs were defined by merging DMLs by t-statistic (611, 613).

7.3.4. Hypermethylated DMRs in low versus high risk disease

367 DMRs were differentially hypermethylated in low versus high risk disease. ≥ 10 5-mC sites were observed in 34 DMRs.

TRAF3 harboured the most 5-mC sites (n=19) within its DMR, with a width of 121 base pairs (bp). Hepatocyte TRAF3 interacts with TAK1 enhancing the activation of downstream JNK and NF-κB cascades promoting insulin resistance, gluconeogenesis, inflammatory response and lipid accumulation in the liver (614). This ***lipid metabolism*** gene is appropriately hypermethylated and presumably downregulated in the low risk group compared to the high-risk group.

BCAT1 was the longest hypermethylated DMR, with a width of 888 bps, containing nine 5-mC sites. BCAT1 catalyses the conversion of alpha-ketoglutarate to glutamate and has been linked to the presence and severity of NAFLD (615-617). Differential DNA methylation at

multiple *BCAT1* loci was reported where hypomethylation and overexpression was associated with adverse clinical events in advanced NAFLD (618). This ***glucose metabolism*** gene is appropriately hypermethylated and presumably downregulated in low risk disease compared to high risk disease.

BSG is the DMR with the greatest mean difference between low and high risk groups (76% methylation in LRR vs. 20% in HR; areastat = 11.7, three 5-mC sites). Shi et al. demonstrated that CD147(BSG/Basigin) expression promotes hepatic fibrogenesis by CD147-induced CXCL1 expression and consequent HSC activation. This ***fibrosis*** related gene is appropriately hypermethylated and presumably downregulated in the low risk group compared to the high-risk group.

In this study, DMRs were ranked by "areaStat" which is the sum of the t-statistics in each DMR. Its value is based on weighting the DMRs by the number of CpGs and not by genomic length. The top hypermethylated DMRs (presumably associated with gene suppression in low risk NAFLD) are listed in **table 7.3.** and includes 2 pseudogenes (MCTS2P, RNA5SP155) (619). To validate our associations, the gene names were referenced to "Harmonizome"- a Comparative Toxicogenomics Database; Gene-Disease Associations dataset, cataloguing 11463 genes associated with NAFLD. All genes were present excluding MCT2SP, RNA5SP155, CD300A and EOGT (620).

Genes significant in **fibrosis** included HM13(620, 621) , CGGBP1 (621, 622), TET1(623), CD300A (624) and RCN 1(625).

Genes significant in **metabolic homeostasis** included EOGT (626) and PIEZO2(627).

Table 7.3. Top 10 hypermethlyated DMRs according to area stat.	
DMR	**Area Stat**
MCTS2P	52.05860201
HM13	52.05860201
CGGBP1	42.30066421
TET1	40.94143642
RNA5SP155	40.66544076
FBLL1	40.13699822
CD300A	38.7313966
RCN1	38.04068378
PIEZO2	37.87158883
EOGT	37.75609473
MCTS2P,Malignant T cell amplified sequence 2; HM13, Minor histocompatibility antigen H13; CGGBP1, CGG triplet repeat-binding protein 1; TET1, Tet Methylcytosine Dioxygenase 1; RNA5SP155, RNA, 5S Ribosomal Pseudogene 155; FBLL1, Fibrillarin Like 1; CD300A, Cluster of Differentiation 300A; RCN 1, Reticulocalbin 1; PIEZO 2, Piezo Type Mechanosensitive Ion Channel Component 2; EOGT, EGF Domain Specific O-Linked N-Acetylglucosamine Transferase	

7.3.5. Hypomethylated DMRs in low vs high risk disease

289 DMRs were significantly hypomethylated low risk disease. $\geq$ 10 5-mC sites were observed in 40 DMRs.

MYO1C harboured the most 5-mC sites (n=11) within its DMR, with a width of 205 base pairs. A recent study suggests that MYO1C and NEMO (nuclear factor κB essential modulator) are responsible for the mechanism of TNF-α–induced insulin resistance. NEMO and MYO1C stimulate TNF-α–induced Ser137 phosphorylation of IRS-1, resulting in the attenuation of insulin signalling and glucose transport (628). This ***metabolic syndrome*** gene is hypomethylated and presumably active in low risk disease compared to high risk disease.

SDHAF4 was the longest hypomethylated DMR, with a width of 785 bps, containing 11 5-mC sites. SDHAF4 protein family is involved in succinate dehydrogenase (SDH) assembly. SDH occupies a central place in cellular energy production, linking the tricarboxylic cycle with the electron transport chain (629). A recent publication suggests a connection between the role of SDHAF4 in succinate dehydrogenase assembly and oxidative stress resistance hypothesising that SDHAF4 is protective against ROS toxicity (630). ROS toxicity is fundamental to NAFLD pathogenesis and this ***glucose metabolism gene*** is appropriately hypomethylated and presumably active in the low risk versus high risk groups.

BHLHE41 was the hypomethylated DMR with the greatest mean difference between the 2 groups (33% methylation in LR vs. 81% in HR; areaStat = -12.99, 4 5-mC sites). A recent review article describes how the circadian clock circuitry interacts with pathways involved in NASH progression. For example, Dec2 modifies the interaction of retinoid X receptor (RXR)α with RXR nuclear receptors, impacting other transcriptional networks that enrich lipid and glucose metabolic pathways (631). This gene affects ***lipid and glucose metabolism*** and is presumably active in low risk disease compared to high risk disease.

Top 10 hypomethylated DMRs were ranked according to areaStat and included 1 pseudogene, A2MP1 are shown in ***table 7.4***

Genes significant in ***fibrosis*** included BMP4 (632), EPN1 (633), DPP9 (634), ADRA2A (635), HGFAC (636).

Genes significant in ***metabolic homeostasis*** included SDHAF4 (630) and SDK 1 (637)

Table 7.4. Top 10 hypomethlyated DMRs according to area stat

DMR	Area Stat
BMP4	-48.00504855
EPN1	-46.62005719
DPP9	-43.18738736
ADRA2A	-43.11661679
SDHAF4	-42.40265087
HGFAC	-40.88100351
SDK1	-40.50285308
A2MP1	-39.54740268
LINC00987	-39.54740268
C1QTNF8	-38.93825423

BMP4, Bone Morphogenetic Protein 4; EPN1, Epsin 1; DPP9, Dipeptidyl Peptidase 9; ADRA2A, Adrenoceptor Alpha 2A**;** SDHAF4, Succinate dehydrogenase assembly factor 4; HGFAC, Hepatocyte growth factor activator; SDK1, Sidekick Cell Adhesion Molecule 1; A2MP1, Alpha-2-Macroglobulin Pseudogene 1; LINC00987, Long Intergenic Non-Protein Coding RNA 987; C1QTNF8, Complement C1q tumour necrosis factor-related protein 8

7.3.6. DNA methylation in low and high risk NAFLD patients and underlying pathophysiology

Polygenic susceptibility in NAFLD as opposed to single gene regulation is responsible for its trademark complex disease characteristics, notably non-linear disease progression. Consequently, insights into disease pathogenesis are more reliably obtained from network based approaches that identify functionally related genes. Phenotype specific NAFLD DMRs in liver biopsy tissue, may also prove to be more accurate for diagnosis of fibrosis stage than

standard histology as studies have shown that DNA methylation occurs independent of anatomical location therefore is less vulnerable to sampling error (167).

DAVID Bioinformatics Resources (DAVID) at http://david.abcc.ncifcrf.gov is an integrated biological analytical resource powered to systematically extract biological meaning from omics data.

Serial pathway mining tools employed within DAVID include Gene Functional Classification and Functional Annotation Chart or Clustering; which were explored for each gene list associated with hypo and hypermethylated DMRs (610)

7.3.6.1. Functional enrichment analysis of hypermethylated DMRs in low vs high risk disease

7.3.6.1.1. Step 1: DAVID Gene Functional Classification

This analysis condenses the gene list (n=268) into biologically meaningful modules to transform a gene-centric analysis to a biological module-centric analysis(610). The functional group with the highest enrichment score (3.26) was reported.

Genes in this group share common biological functions including roles in ***fibrosis*** (LLGL2 (638)), ***metabolic homeostasis*** (AAMP(639), TBL3 (640), KCT3 (641)) and ***carcinogenesis*** (WDR1 (642), ATG16L2 (643), WDR 12 (644),DMXL1 (645)) **(Table 7.5)**

Table 7.5. Gene Functional Classification	
Gene Group 1	**Enrichment score 3.26**
LLGL2	LLGL2, scribble cell polarity complex component(LLGL2)
AAMP	Angio associated migratory cell protein(AAMP)
WDR1	WD repeat domain 1(WDR1)
ATG16L2	autophagy related 16 like 2(ATG16L2)
WDR78	WD repeat domain 78(WDR78)
WDR12	WD repeat domain 12(WDR12)
TBL3	Transducin beta like 3(TBL3)
KCTD3	Potassium channel tetramerization domain containing 3(KCTD3)
DMXL1	Dmx like 1(DMXL1)

7.3.6.1.2. Step 2: DAVID Functional Annotation Chart

Functional Annotation Chart platform provides a gene-term enrichment analysis to identify the most relevant over-represented biological terms associated with the 268 hypermethylated gene list. A filter was applied to consider the biological applications of GOTERM categories only (646, 647). The top 10 GOTERM annotations are shown in ***table 7.6***

Table 7.6. DAVID Functional Annotation chart				
Category	**Term**	**Count**	**%**	**P-Value**
GOTERM_MF_DIRECT	Protein binding	127	65.5	3.80E-07
GOTERM_CC_DIRECT	Cytoplasm	85	43.8	2.50E-06
GOTERM_CC_DIRECT	Nucleoplasm	53	27.3	1.10E-05
GOTERM_CC_DIRECT	Nucleus	81	41.8	1.40E-04
GOTERM_BP_DIRECT	Chromatin organization	5	2.6	1.10E-03
GOTERM_BP_DIRECT	Activation of phospholipase C activity	4	2.1	2.60E-03
GOTERM_BP_DIRECT	Positive regulation of fibroblast proliferation	5	2.6	2.70E-03
GOTERM_CC_DIRECT	Cytosol	49	25.3	8.20E-03
GOTERM_BP_DIRECT	Wnt signalling pathway, calcium modulating pathway	4	2.1	8.30E-03
GOTERM_BP_DIRECT	Transcription, DNA-templated	33	17	9.60E-03

The over-represented terms are associated with pathways relevant to fibrosis progression (wnt signalling, fibroblast proliferation) in NAFLD. Other terms are linked to non-specific signal transduction pathways and cellular metabolism. To facilitate biological interpretation of the GO annotations in a network context, KEGG pathway analysis were also considered. Pathways enriched included the Wnt signalling pathway, Hippo signalling pathway, vascular smooth muscle contraction, aldosterone synthesis and the Notch signalling pathway, all of which are closely associated with ***fibrosis progression*** (648-652)

Table 7.7. KEGG Pathway Analysis				
Category	**Term**	**Count**	**%**	**P-Value**
KEGG_PATHWAY	Wnt signalling pathway	7	0	3.80E-03
KEGG_PATHWAY	Pathways in cancer	11	0	9.80E-03
KEGG_PATHWAY	Hippo signalling pathway	6	0	2.40E-02
KEGG_PATHWAY	Vascular smooth muscle contraction	5	0	3.80E-02
KEGG_PATHWAY	Aldosterone synthesis and secretion	4	0	5.80E-02
KEGG_PATHWAY	Melanogenesis	4	0	9.50E-02
KEGG_PATHWAY	Notch signalling pathway	3	0	9.60E-02

7.3.6.1.3. DAVID Functional Annotation Clustering

Functional Annotation Clustering uses a clustering concept that measures relationships among the annotation terms based on the degree of their co-association with genes within the list in order to cluster highly similar annotations into functional annotation groups (610). The annotation cluster with the highest enrichment score was reported. Each annotation feature in the cluster was inserted into the Reactome database and associated functional pathways are reported in column 4 of ***table 7.8***. Pathways again focused on themes pivotal to ***fibrogenesis, regeneration***, ***wound healing*** and ***carcinogenesis***

Table 7.8. DAVID Functional Annotation clustering				
Annotation Cluster 1	**Enrichment Score 2.78**	**Count**	**P-Value**	**Pathway**
UP_SEQ_FEATURE	repeat: WD 3	12	3.10E-04	Major pathway of rRNA processing in the nucleolus and cytosol
UP_SEQ_FEATURE	repeat: WD 2	12	4.10E-04	Deubiquitination
UP_SEQ_FEATURE	repeat: WD 1	12	4.10E-04	Response to elevated platelet cytosolic Ca2+
UP_KEYWORDS	WD repeat	12	4.50E-04	
UP_SEQ_FEATURE	repeat: WD 5	11	4.60E-04	Chromatin organization
UP_SEQ_FEATURE	repeat: WD 8	6	6.40E-04	RAF/MAP kinase cascade
INTERPRO	WD40 repeat,	9	7.40E-04	VEGFA-VEGFR2 Pathway
UP_SEQ_FEATURE	repeat: WD 4	11	8.00E-04	tRNA modification in the nucleus and cytosol
INTERPRO	WD40-repeat-containing domain	12	9.50E-04	VEGFA-VEGFR2 Pathway
INTERPRO	WD40 repeat	11	1.20E-03	VEGFA-VEGFR2 Pathway
INTERPRO	WD40/YVTN repeat-like-containing domain	12	1.80E-03	VEGFA-VEGFR2 Pathway
UP_SEQ_FEATURE	repeat: WD 7	8	1.90E-03	Chromatin organization
SMART	WD40	11	2.00E-03	
UP_SEQ_FEATURE	repeat: WD 6	9	2.10E-03	Cilium Assembly
UP_SEQ_FEATURE	repeat: WD 11	4	4.90E-03	
UP_SEQ_FEATURE	repeat: WD 10	4	5.40E-03	
UP_SEQ_FEATURE	repeat: WD 9	4	1.40E-02	Caspase activation via Dependence Receptors in the absence of ligand
INTERPRO	G-protein beta WD-40 repeat	5	2.30E-02	Cell junction organization
UP_SEQ_FEATURE	repeat: WD 12	3	2.60E-02	Major pathway of rRNA processing in the nucleolus and cytosol

7.3.6.2. Functional enrichment analysis of hypomethylated DMRs in the low vs high risk disease

DMRs hypomethylated in the low risk group (n=207) were subject to similar functional category overrepresentation and pathway analysis.

7.3.6.2.1. Step 1: DAVID Gene Functional Classification

The hypomethylated DMR gene list (n=207) was condensed into 5 functional groups with genes that share a common biological function. The gene group with the highest enrichment score (1.65) was reported **(Table 7.9).** The listed genes are uniformly themed around ***adipogenesis*** - NR2F6 (653), ESRRG (654), RORC (655) and ESRRA (656)

Table 7.9. Gene Functional Classification	
Gene Group 1	***Enrichment score 1.65***
NR2F6	Nuclear receptor subfamily 2 group F member 6(NR2F6)
ESRRG	Estrogen related receptor gamma(ESRRG)
RORC	RAR related orphan receptor C(RORC)
ESRRA	Estrogen related receptor alpha(ESRRA)

7.3.6.2.2. Step 2: DAVID Functional Annotation Chart

Functional Annotation Chart facilitates functional category overrepresentation analysis generating a list of the over-represented biological terms associated with the 207 hypomethylated gene list. The top 10 GOTERM categories are shown in ***table 7.10***

Table 7.10. DAVID Functional Annotation chart				
Category	**Term**	**Count**	**%**	**P-Value**
GOTERM_BP_DIRECT	Steroid hormone mediated signalling pathway	6	3.1	1.80E-04
GOTERM_BP_DIRECT	Mitophagy	8	4.1	1.60E-03
GOTERM_BP_DIRECT	Regulation of transcription, DNA-templated	5	2.6	1.90E-03
GOTERM_BP_DIRECT	Circadian regulation of gene expression	4	2.1	4.10E-03
GOTERM_BP_DIRECT	Activation of MAPK activity	4	2.1	4.70E-03
GOTERM_BP_DIRECT	Beta-catenin destruction complex disassembly	3	1.5	9.20E-03
GOTERM_BP_DIRECT	Positive regulation of cellular response to insulin stimulus	24	12.3	1.10E-02
GOTERM_BP_DIRECT	Platelet-derived growth factor receptor signalling pathway	38	19.5	1.10E-02
GOTERM_BP_DIRECT	Negative regulation of insulin secretion	15	7.7	1.10E-02
GOTERM_BP_DIRECT	Autophagy	43	22.1	1.50E-02
GOTERM_BP_DIRECT	Transcription, DNA-templated	4	2.1	1.50E-02

This spectrum of GOTERMS cover gene functions that are well characterised in NAFLD liver disease.

Enriched Pathways associated with fibrosis; The ***MAPK pathway*** is associated with fibrosis, modulating JNK associated hepatocyte cell death and metabolism (657).

BCL-B is well characterised in the regulation of ***mitophagy*** in HSC during liver fibrosis regression (658). ***Wnt/β-catenin*** signalling appears important in normal wound healing and its sustained activation is associated with fibrogenesis (659). Clinical studies have confirmed that excessive activation of ***PDGF*** and its downstream molecules appears to be associated with the extent of necroinflammation and fibrosis (660). ***Autophagy*** in chronic liver disease is now thought to be protective and is being developed as a therapeutic target for fibrosis (661).

Enriched pathways associated with metabolic homeostasis;

Well characterised associations exist between the ***circadian clock*** and the metabolism of NAFLD (662).

While all these associations are plausible in NAFLD pathogenesis, KEGG pathway analysis were more in keeping with the overall derangement in adipogenesis observed in the preliminary gene functional classification report ***table 7.11***. KEGG pathways enriched included the neurotrophin-1/B-cell- factor-3 signalling pathway (663) and the amino acid and sugar metabolism pathways, whose irregularities are likely responsible for the metabolic dysfunction observed in NAFLD (664)

Table 7.11. KEGG Pathway Analysis

Category	Term	Count	%	P-Value
KEGG_PATHWAY	Neurotrophin signalling pathway	5	2.6	2.60E-02
KEGG_PATHWAY	Amino sugar and nucleotide sugar metabolism	3	1.5	7.50E-02

7.3.6.2.3. DAVID Functional Annotation Clustering

Functional Annotation Clustering condensed the hypomethylated gene list into highly similar functional annotation groups (610). The functional annotation group with the highest enrichment score was reported in ***table 7.12***

Table 7.12 DAVID Functional Annotation clustering			
Annotation Cluster 1	**Enrichment Score 2.78**	**Count**	**P-Value**
GOTERM_BP_DIRECT	Steroid hormone mediated signalling pathway	6	1.80E-04
UP_SEQ_FEATURE	Zinc finger region: NR C4-type	5	6.30E-04
UP_SEQ_FEATURE	DNA-binding region: Nuclear receptor	5	6.30E-04
INTERPRO	Zinc finger, nuclear hormone receptor-type	5	8.80E-04
INTERPRO	Steroid hormone receptor	5	9.50E-04
INTERPRO	Nuclear hormone receptor, ligand-binding, core	5	1.00E-03
SMART	ZnF_C4	5	1.40E-03
SMART	HOLI	5	1.60E-03
GOTERM_MF_DIRECT	Steroid hormone receptor activity	5	1.90E-03
INTERPRO	Zinc finger, NHR/GATA-type	5	2.10E-03
GOTERM_MF_DIRECT	RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding	4	4.70E-03
GOTERM_BP_DIRECT	intracellular receptor signalling pathway	3	4.80E-02
GOTERM_BP_DIRECT	transcription initiation from RNA polymerase II promoter	5	5.20E-02
GOTERM_MF_DIRECT	transcription factor activity, sequence-specific DNA binding	11	4.20E-01
GOTERM_MF_DIRECT	sequence-specific DNA binding	6	5.40E-01
UP_KEYWORDS	Activator	6	6.90E-01

The annotation terms with immediately recognisable roles in NAFLD fibrogenesis include Zinc finger proteins (665), steroid hormone receptor–ligand interactions and associated intracellular signalling pathways (e.g. the renin–angiotensin–aldosterone system and endothelin-, farnesoid X receptor (FXR) or PPAR-associated pathways) which are important for modulating ***fibrosis progression*** and underpin ***metabolic abnormalities*** in NASH (666).

7.4. DISCUSSION

NAFLD is a complex disease trait with marked interpatient variation in disease progression. From a clinical perspective, NAFLD stratifies into two poorly defined clinical courses. For the majority of patients, NAFLD is a low risk, non-progressive disease however an underreported cohort experience high risk progressive disease. In a local paired biopsy study with a median follow-up of 6.6 years; 42% patients had fibrosis progression while 58% remained static or regressed. Of concern, 6 of 27 (22%) patients with baseline NAFL, progressed to stage 3 fibrosis during the study period (53).

7.4.1. Solid platform for establishment of a progression signature

Early stratification of high risk NAFLD is of clear clinical importance in establishing those most likely to derive benefit from the soon to be FDA approved anti-fibrotic therapies. The efficacy of NAFLD therapies will likely be intrinsically linked to their timing of use. Therapy in NAFLD will most certainly be subject to "therapy sequencing" a concept observed in PBC, where NAFLD patients will likely be offered a considerable trial of lifestyle adjustments before progressing to anti-fibrotic therapies (667), which for some patients (rapid progressors) may lead to an unnecessary delay in treatment initiation. As alluded to in the introduction, newer sequencing and analysis platforms provide a rich environment for the development of prognostic assays. This proof of concept study sought to characterise differential DNA methylation between those with low and high risk NAFLD in archival FFPE tissue obtained at the earliest point in the disease course (F0/F1 fibrosis). The historic UK-DELTA cohort were well characterised and at study onset and had confirmed disease outcomes allowing accurate phenotyping of both low and high risk disease. The characterisation of a NAFLD methylation signatures at baseline to distinguish between patients with high and low risk disease that are biologically distinct from early in the disease process will be valuable in precision medicine and may serve as treatment targets for high risk disease.

7.4.2. Current Indirect "prognostic" biomarkers: Useful but not fit for purpose

Clinicians to date have classified 'high risk' NAFLD as those diagnosed with advanced fibrosis on biopsy. Only a few studies have attempted de novo biomarker development by using

omics approaches and biomarkers developed in this forum have largely been diagnostic for advanced fibrosis only. This is thought to be the first epigenetic study powered solely to characterise an early "prognostic" fibrosis biomarker in NAFLD.

Milestones in NAFLD biomarkers are summarised and include;

(1) Clinical risk factors: Going back to first principles, in the as yet unpublished EuroDELTA paired biopsy study paper, 7% of patients exhibited rates of fibrosis progression exceeding 0.5 stages per year. These more rapid progressors tended to have a higher NFS at baseline than those that did not progress or only progressed slowly, probably because NFS incorporates a number of features that have been associated with risk of fibrosis progression (e.g. higher BMI and presence of T2DM). However, neither BMI nor T2DM alone had adequate prognostic power.

(2) Genetics: The non-synonymous *PNPLA3* (rs738409), *TM6SF2* (rs58542926) and MBOAT7 single nucleotide polymorphisms (SNPs) (201), first linked with NAFLD by genome-wide association studies, are associated with an increased risk of steatohepatitis and more severe liver fibrosis (202-207, 668). However, in predictive tests, they never succeeded to enhance diagnostic accuracy by >1% (450, 520). All patients in this study cohort were genotyped for PNPLA3. 2 patients (1 in the high risk and 1 in the low risk group) were heterozygous for G.

(3) Epigenetic biomarkers: in house efforts to date have characterised methylation signatures derived from NAFLD liver biopsy genomic DNA and plasma ccfDNA to differentiate mild from severe fibrosis and have suggested "diagnostic" biomarkers which require further independent validation (167, 168).

(4) Transcriptomics - mRNAs: a recent study (2018) examining miRNAs previously described as predictive (n=18) proceeded to validate 9 as predictive of NAFLD severity. Again, this biomarker has been developed within a "diagnostic" context of use (669).

(5) Proteomics: A recent proof-of concept study demonstrated that fibrogenesis flux rates both in liver tissue and blood can be used to identify rapidly progressing disease based on mass spectrometry quantification of liver collagen fractional synthesis rate (FSR) yet this qualifies as a biomarker with a "monitoring" context of use as opposed to "prognostic" context of use (670).

The forum therefore remains open for integrating OMICs with clinical data and the development of a NAFLD prognostic biomarker.

7.4.3. Importance of detecting "high" risk NAFLD

To date there has been a collection of studies establishing a robust association between increased mortality in patients with fibrosis progression, irrespective if NASH was present or not in the baseline liver biopsy and after adjustment for confounders (55, 56, 671). Of increasing concern is the population with lean NAFLD. In a recent study (n=646, mean follow-up 19.9 years) by Hagstrom at al, they found that lean NAFLD patients (19% of cohort) had an increased risk of severe liver disease despite a lower prevalence of advanced fibrosis and NASH at baseline. A prognostic signature will be particularly valuable in this patient subgroup as this study would suggest that that fibrosis progression is faster in lean NAFLD than in patients with NAFLD with a higher BMI contradicting currently accepted clinical dogmas.

7.4.4. Signature characterisation summary

This study used NGS to map the entire NAFLD genomic DNA methylome of a well characterised cohort of NAFLD patients; stratified into 2 phenotypes- stable/regressors or fast progressors as described in NAFLD natural history studies. It was found that genomic DNA harbours over 60,000 single nucleotide methylation signatures and has enormous potential to prognosticate NAFLD fibrosis. On initial perusal, the observed signatures were appropriately associated with pathways and genes characterised in fibrosis progression and metabolic homeostasis. Single nucleotide resolution analysis was not reported in this thesis in favour of regional differential methylation analysis which is more likely to be associated with transcriptional gene control.

DMR characterisation the majority of selected DMRs were located at functional genomic regions thus were more likely to be involved in regulation of gene expression. The number of hypermethylated DMRs was not substantially different from that of hypomethylated DMRs (367 versus 289) suggesting that both up and down regulation of DNA methylation is involved in low and risk disease reflective of other reports in the literature showing mixed gene expression in advanced fibrosis (672).

Hypermethylated DMR characterisation. The most significant and largest DMRs were associated with genes uniformly involved with metabolic homeostasis, while genes associated with the top 10 hypermethylated DMRs revealed that 50% of them were well characterised in pathways controlling liver fibrosis. When ranked based on greatest mean difference thus potential discriminatory capacity, there was a 50/50 split between genes associated with fibrosis and dysregulated metabolism.

Hypomethylated DMR characterisation. The most significant and largest DMRs were associated with genes involved in metabolic homeostasis, while in the top 10 genes 50% had well characterised roles in fibrosis progression while only 2 genes were associated with metabolic dysfunction. When ranked based on greatest mean differences 3 (30%) were associated with fibrosis while 50% were linked to metabolic dysregulation.

Based on DMR ranking by significance, size and magnitude of difference, signatures were suggested. However, the novel DMRs characterised were interrelated in terms of biological processes and pathways and based on the small sample size it was not possible to ascribe specific contexts to DMRs as hypo/hypermethylation being associated with liver fibrosis or metabolic homeostasis exclusively.

7.4.5. Comparison of prognostic DMRs in this study with diagnostic DMRs in mild and severe NAFLD and WGS studies

In a thesis submitted by TH, NGS was used to sequence the entire plasma DNA methylome of a well-characterised prospective cohort of patients with biopsy-proven NAFLD (14 mild (F0-F2 fibrosis), 12 severe cases (F3-F4 fibrosis)(563). 251 DMRs were significantly hypermethylated in severe fibrosis compared to mild fibrosis and 248 DMRs were significantly hypomethylated between mild vs. severe fibrosis. When compared, the top 10 most significant genes from that study (based on areaStat and mean difference) did not overlap with the DMRs gene lists in this study. This was not surprising as the study conducted by TH was powered from a diagnostic perspective and included histological and plasma specimens with both mild and advanced disease. It is also possible that plasma ccfDNA may contain non-liver specific DMRs which can be significantly influenced by liver-unrelated processes (e.g. haemolysis).

A study by conducted in Duke University was the first WES investigation of genetic variation in NAFLD fibrosis. The objectives were similar to this study where NAFLD patients were divided into extreme phenotypes based on liver fibrosis stage and clinical risk factors to investigate rare variants that might predispose to or protect from advanced NAFLD fibrosis-akin to a NAFLD progression signature. Two extreme phenotypes of NAFLD: protective (n=54) and progressor (n=54) were defined based on the development of advanced liver fibrosis (fibrosis stage, F3-F4). The protective phenotype included NAFLD patients expected to have significant liver injury and fibrosis based on clinical risk factors, but with no or little fibrosis on liver biopsy. At the other extreme, the progressor phenotype included NAFLD patients expected to have little fibrosis based on a lack of clinical risk factors but biopsy showed advanced liver fibrosis or cirrhosis (599). In our study, TET1 was one of the top 10 hypermethylated DMRs in low risk disease. In the study conducted in Duke University, in the progressor (n-=54) versus population control (n=4455), (although a non-significant gene-based association) under recessive single-variant and gene-based models, a single nonsynonymous variant, T87S (rs140677396, *P*= 1.76E-04 single-variant) was enriched in TET1. Classifying the role of TET1 in NAFLD, Pirola et al. used targeted next-generation sequencing to explore the contribution of genetic variations within *TET* loci of relevance to NAFLD. Analysis of missense variants in *TET1* revealed a putative role for the *TET1* locus in the modulation of apoptosis and liver injury in NAFLD(553). WGS in this study was conducted on FFPE genomic liver DNA as opposed to genomic DNA subject to LCM in our study therefore the gene list detected may contain non-liver specific signals.

7.4.6. Clues to pathogenesis in low and high risk NAFLD

A clear difference has been demonstrated between the high and low risk NAFLD phenotype from early stages of disease. This may suggest that low and high risk NAFLD represent two distinct, yet interrelated disease processes.

Low and high risk disease characteristics

1. insights obtained from functional enrichment analysis of DMRs hypermethylated in low versus high risk disease; "Gene functional classification" grouped the gene list into biological modules. The module with the highest enrichment score, as expected involved diverse pathways associated with fibrosis, metabolic homeostasis and carcinogenesis. The functional annotation chart refined this further and the suggested KEGG pathways were

more reflective of enrichment of pathways involved in ***liver fibrogenesis*** (Wnt signalling pathway, Hippo signalling pathway, vascular smooth muscle contraction, Aldosterone synthesis and the Notch signalling pathway) as opposed to carcinogenesis or metabolic dysregulation. It is possible to speculate that hypermethylation (gene suppression) in low risk disease versus high risk disease, would suggest potential downregulation of the fibrogenic process in the low risk group.

2. Insights obtained from functional enrichment analysis of DMRs hypomethylated in low versus high risk disease; The "Gene functional classification" module with the highest enrichment score involved pathways uniformly associated with adipogenesis. The "functional annotation chart" suggested KEGG pathways continued on the theme of metabolic homeostasis derangement involving the neurotrophin-1/B-cell- factor-3 signalling pathway (663) and the amino acid and nucleotide sugar metabolism pathways contributing to metabolic dysfunction in NAFLD (664).

This study is not powered to confer causality; however, it is possible to hypothesis that in low risk disease; pathways ***associated with liver fibrosis*** are loosely associated with hypermethylated DMRs and are in theory are globally repressed and downregulated. In contrast, pathways associated with ***metabolic homeostasis*** are associated with hypomethylated DMRs and are in theory globally activated in low risk NAFLD. If true, this may suggest that the low risk NAFLD phenotype may benefit from more stringent management of their metabolic syndrome as opposed to the novel anti-fibrotic therapies soon to be FDA approved, with the opposite holding true for high risk group.

7.4.7. Study strengths and limitations

Reference standard; the pros and cons of liver biopsy as a reference standard has been discussed in previous chapters as a study limitation. However, as the current gold standard it was the most accurate way to categorise the historic UK-Delta cohort into the correct phenotypes.

Study design; this was a single centre study set in a tertiary referral centre therefore was associated with potential referral bias.

Sample size; small sample size is a limitation in this study. The consequences of low statistical power include overestimation of effect size and low reproducibility of results with a reduced chance of detecting a true effect. Relating to the NGS analysis, it is possible that additional DMRs that could be highly specific as prognostic signatures were overlooked as a result of the small cohort chosen or the depth of sequencing performed in the study. The study cohort was small in numbers, due to the feasibility of having sufficient specimen meeting the inclusion criteria. A limited number of patient biopsies had F0/F1 biopsies as their index biopsies. In line with the advancement of non-invasive screening methods to assist the liver biopsy referral process, mild disease patients are now appropriately seldom biopsied. However, the decision for a small sample size was considered appropriate as this was a pilot study where we wanted to ascertain if differences existed between high and low risk disease before embarking on a large, well-phenotyped follow-up study.

Novel study; this is proposed as the first WGBS investigation of epigenetic variation in the distinct sub-groups of low and high risk NAFLD from the earliest disease state (F0/F1 fibrosis). It is also the first study to extract liver biopsy genomic DNA from liver biopsy tissue subject to LCM to minimise cellular contamination from non-hepatocytes. This allowed us to infer that any observed differences in DNA methylation density are not reflective of non-specific cellular or architectural changes inherent in the fibrogenic process.

Use of NGS in this study is a more powerful platform, as opposed to microarray profiling which provides coverage of a much smaller area of the genome.

Study Cohort: Gold standard liver biopsy–confirmed NAFLD was used to ensure accurate histologic phenotyping of patients. This study was unique in that unlike other studies, predicting high and low risk disease we employed a paired biopsy cohort that was accurately phenotyped and included baseline biopsies only. Furthermore, cohorts were similar in terms of clinical factors known to influence disease progression, up to an including PNPLA3 genotype. Most patients had stages 0, 1, and 3 fibrosis, therefore it was not possible to identify extremes with just isolated steatosis or cirrhosis. The study also did not include population controls representative of the general population, viral or autoimmune liver disease and therefore had limited power and generalisability.

Proof of concept study only; the results presented are at best associations and cannot be used to imply causality; but do provide a basis for future research

7.4.8. Future directions for study

Future validation: In this study, multiple associations in genes linked to metabolic dysregulation and dysregulated ECM turnover in low and high risk NAFLD have been suggested. These findings are important for future hypothesis-driven research but require replication in independent NAFLD cohorts, as metabolic and wound healing genes are so diverse and non-specific and compose a substantial fraction of the human genome. Looking at the entire spectrum of DMRs, multiple DMRs were differentially methylated in Wnt signalling (9 DMRs in total: 2 hypo- CSNK1A1, LRP5 and 7 hyper- FZD4, APC2, PLCB2, TBL1XR1, TCF7L1, WNT5 and ABMP4) which could conceivably reflect disruption of this pathway in NAFLD potentially involving both fibrosis progression and liver regeneration in low and high risk disease (673). Further clarification is necessary.

Cognisant of the 'reproducibility crisis' intricate to bioinformatic analysis, a repeat analysis, with a focus on a more complex regression model to select DMRs (e.g. BiSeq) may be performed to see if it yields similar results.

This study suggests that low and high risk NAFLD may represent distinct disease subtypes accounting for the non-linear nature of disease progression. Future large scale longitudinal studies are needed to validate the clinical utility of the proposed prognostic DNA methylation signatures to detect those with both low and high risk disease and develop a molecular signature unique to each NAFLD patient that will dictate suitable follow-up regimens, treatment options (lifestyle versus therapeutics) and HCC risk.

This study employed a small fraction of the UK Delta cohort (n=108), This cohort has now been expanded and includes samples from other members of the EpoS consortium now comprising the EuroDelta (n=526) cohort for future studies.

7.5. CONCLUSION

The results obtained in this study are proof of concept only, with research in this niche area remaining undeveloped. The proposed signatures can become useful prognostic biomarkers for the diagnosis of both low and high risk NAFLD patients. Identifying high-risk patients at an early stage will enable swift intervention as novel therapeutics come to trial. Early stratification will also enable assignment of low-risk patients to primary care follow-up thereby reducing the burden on more expensive specialist resources.

CHAPTER 8.

GENERAL DISCUSSION AND CONCLUSION

Non-alcoholic fatty liver disease (NAFLD) was first described as a distinct clinical entity four decades ago. It has now gained notoriety in the field of hepatology due to its high prevalence and contributions to the burden of end-stage liver disease and cardio-metabolic mortality.

The initial studies in this thesis are concerned with the QoL and economic burden of NAFLD while the remaining areas of study were focused on improving NAFLD patient care delivery by improving the diagnosis of fibrosis in NAFLD which has been established as the key histological determinant of disease related mortality. The areas covered, although diverse are inter-related. NAFLD is hypothesised to lead to QoL impairment in addition to well documented increases in morbidity and mortality. This thesis sought to examine this in a UK cohort. Improving care delivery in NAFLD is central to improving patient QoL. The key method by which to achieve this, as for any disease is improved treatment. To date, the lack of non-invasive fibrosis biomarkers has impeded the diagnosis, risk stratification and monitoring of patients so that a large portion are presenting with advanced disease. This has also impeded clinical trial recruitment and retention which still depends on histological effect as an endpoint. Thus, NAFLD fibrosis biomarker and prognostic signature development was one of the objectives. A cost of illness study was also performed as part of this thesis as they provide useful information assessing opportunity and monetary costs associated with health care interventions. As a number of phase III clinical trials in NAFLD draw to an end, this data will be valuable in performing cost-effectiveness analysis of new potentially QoL improving drugs. Given the good oral tolerability of imminent drugs, it is likely that cost will be the principal barrier to their widespread adoption.

Firstly, we established the NAFLD disease burden in a UK setting. NAFLD patients suffer from significant impairment in quality of life, particularly in relation to fatigue, while their mental health appears to be less significantly affected. A variety of demographic, clinical states and biological factors were investigated as causative agents however, the most significant contribution to impaired QoL in NAFLD was patient perceived fatigue. Prominent liver histological factors in QoL impairment and patient reported fatigue included lobular

inflammation and fibrosis, thus supporting the use of novel anti-fibrotic drugs for QoL improvement. There is a need for large, prospective longitudinal studies powered to delineate QoL correlates and summarise NAFLD patient QoL profiles in parallel to ongoing interventional RCTS in NAFLD. Current phase III trials have a PRO (CLDQ-NASH) to assess efficacy of intervention on QoL and will be informative going forward. Delineation of the factors which drive impaired QoL in NAFLD will permit the development of therapeutic targets and increased awareness of QoL in NAFLD and will allow clinicians to consider both clinical and patient factors in treatment selection (246).

Secondly, in terms of disease burden we looked at it from an economic perspective. This study provides a useful breakdown of costs reflective of suspected and established NAFLD diagnoses. Such disease costing metrics are useful to have generated as it is highly likely that an increase in OPD costs due to NAFLD is on the horizon given that 82% of the suspect NAFLD cases in this study were over the next 11 months confirmed as NAFLD. The large, heterogeneous snapshot of NAFLD and ALD patients is reflective of the case-mix observed in hepatology practices in the UK and provides useful information to guide health care policy makers to decide the most sensible and strategic monetary investments to improve patient care. Multivariate regression analysis in the NAFLD cohort established the number of clinic appointments and the presence of advanced disease as the main cost drivers. The findings generated in this study are in line with current trends reported in the US and in Europe and have shown that NAFLD is associated with considerable costs. Unfortunately, the possibility of a cure for both ALD and NAFLD, both lifestyle associated liver diseases is unlikely. There will inevitably be challenges in many health care systems to support this expansion over the coming years.

Following the initial descriptive socio-economic data, efforts then were concentrated on ways to improve patient care. Given the large burden of NAFLD worldwide, there is a critical need for simple and accurate non-invasive tests to stage liver fibrosis, risk stratify and monitor response to treatment and so improve patient QoL. This is particularly important with the recent development of several new drugs for NAFLD that are going through advanced-phase clinical trials. Currently, there is reliance on liver biopsy to monitor patients in the clinical

trials, but this will not be practical once these drugs are widely used. We looked to validate direct collagen biomarkers from a FDA diagnostic context of use. These collagen biomarkers are currently being assessed by the EMA/FDA for clinical trial pre-screening (PROC3). Recent studies have described the role of epigenetic mechanisms, in particular DNA methylation, may have in fibrosis progression in chronic liver disease. We looked to validate previous in-house work that described novel methylation fibrosis diagnostic signatures to move closer to translating these findings into a potential non-invasive biomarker of liver fibrosis. In an additional novel proof of concept study, we then characterised a DNA methylation signature of high and low risk NAFLD disease. Given the vast interpatient variability in NAFLD this constitutes one of the major unmet needs in NAFLD research and patient management.

We examined a panel of protein based biomarkers for fibrosis in NAFLD. PROC3 demonstrated the most favourable biomarker characteristics and we then performed a dedicated PROC3 investigation study which was the first study to implement a robust methodological approach and that provides a truly independent analysis of PRO-C3 biomarker performance for the diagnostic context of use. The recently Innovative Medicine Initiative (IMI)-granted Liver Investigation: Testing Marker Utility in Steatohepatitis (LITMUS) consortium, which is associated with this study group, is an ideal platform to co-ordinate this research in an academic-industry collaborative effort.

Dr. Timothy Hardy demonstrated that PPARγ promoter was differentially methylated in circulating plasma DNA and could be used to stratify disease severity in NAFLD and alcohol related liver disease. We further validated this in an NAFLD, Hepatitis B and systemic sclerosis cohort establishing this as a potential novel fibrosis biomarker with liver organ specificity. We also looked to validate NAFLD specific targets derived from a methylome map of ccfDNA in NAFLD patient plasma to detect patients with advanced NAFLD. However, further work is needed to validate the selected targets and potential reasons for failure to validate has been discussed. Given the friable nature of ccfDNA, attention in the future may be more appropriately given to another branch of epigenetics, namely miRNAs. miRNAs play an important role in regulating gene expression and, importantly, are released into the extracellular space and body fluids, where they remain remarkably stable. As such,

circulating miRNAs have been investigated in a wide variety of NAFLD and HCC animal models, as well as in a large cohort of patients, holding great potential as robust biomarkers. The most studied circulating miRNA to date is circulating miR-122, which with randomised controlled studies may be a highly accurate diagnostic tool for NAFLD (674-676).

Finally, the last area of this thesis is a study which has generated the first methylome map of low versus high risk disease in NAFLD, using baseline F0/F1 biopsy specimens subject to LCM as a platform to define a methylation progression signature. The findings suggest that high and low risk NAFLD while interrelated, may be biologically distinct from disease onset. The data provided by this proof of concept study has provided insights into disease risk in NAFLD, with the intention that future work will address the function of specific loci and the possibility of a DNA-methylation-based diagnostic test distinguishing those with progressive disease from those with stable disease or regression early in the disease process. Future large scale longitudinal studies are needed to validate the clinical utility of the proposed prognostic DNA methylation signatures to detect those with both low and high risk disease and develop a molecular signature unique to each NAFLD patient that will dictate suitable follow-up regimens, treatment options (lifestyle versus therapeutics) and HCC risk.

Additional studies will also need to confirm whether potential signature DMRs translates to expression changes at RNA level in liver tissue. RNA was also extracted from the FFPE biopsy at time of genomic DNA extraction which will be available for further studies. Finally, the targets discovered in these studies may also be applicable to other fibrotic disease such as kidney, lung or skin fibrosis, and may provide a true fibrosis marker, irrespective of organ specificity.

In summary, the contents of this thesis provide useful insights into the burden associated with NAFLD disease from a UK perspective. It offers strategies to improve patient care through fibrosis biomarker validation and explores novel DNA methylation diagnostic and prognostic signatures in line with the objectives of precision medicine.

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APPENDICES

Appendix A: Health Related quality of life in NAFLD associates with hepatic inflammation

Clinical Gastroenterology and Hepatology 2019;17:2085–2092

Health-related Quality of Life in Nonalcoholic Fatty Liver Disease Associates With Hepatic Inflammation

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BACKGROUND & AIMS: **Chronic liver disease has negative effects on health-related quality of life (HRQL). We analyzed data from the European non-alcoholic fatty liver disease (NAFLD) registry to assess the effects of NAFLD on HRQL.**

METHODS: **We collected data from 304 patients (mean age, 52.3 ± 12.9 years) with histologically defined NAFLD enrolled prospectively into the European NAFLD Registry in Germany, the United Kingdom, and Spain. The chronic liver disease questionnaire (CLDQ) was completed within 6 months of liver biopsy collection.**

RESULTS: **The mean CLDQ overall score was 5.0 ± 1.2, with the lowest score in the category fatigue (4.3 ± 1.6) and the highest scores for activity (5.4 ± 1.4). Women had significantly lower CLDQ scores than men (4.6 ± 1.3 vs 5.3 ± 1.1; $P < .001$). We found negative correlations between CLDQ scores and presence of obesity ($P < .001$), type 2 diabetes ($P < .001$), and dyslipidaemia ($P < .01$). There was a negative correlation between level of aspartate aminotransferase, but not alanine aminotransferase, and HRQL. Higher histological score of steatosis (1 vs 3) resulted in lower mean CLDQ score (5.3 ± 1.1 vs 4.5 ± 1.4; $P < .01$); higher level of lobular inflammation (0 vs 3) also resulted in lower mean CLDQ score (5.3 ± 1.2 vs 3.9 ± 1.8; $P <. 001$). In contrast, advanced fibrosis (F3–4) compared to early or intermediate fibrosis (F0–2) had no significant effect on mean CLDQ score (4.9 ± 1.2 vs 5.1 ± 1.3; $P = .072$). In multivariate analysis, patients sex, age, presence of type 2 diabetes, and inflammation were independently associated with low HRQL.**

CONCLUSION: **In an analysis of data from the European NAFLD registry, we observed a substantial burden of symptoms in patients. In addition to age, sex, and the presence of diabetes, detection of lobular inflammation in biopsies correlated with lower HRQL.**

Keywords: Nonalcoholic Steatohepatitis; Patient-Reported Outcomes; Cirrhosis; Emotional Function.

See editorial on page 1950; see related article on page 2093 in this issue of *Clinical Gastroenterology and Hepatology*.

Nonalcoholic fatty liver disease (NAFLD) is the fastest growing and most common cause of liver disease globally.[1] It is estimated to affect up to 30% of the population, and a continued increase has been predicted in the coming years.[2] Distinction between NAFLD

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Abbreviations used in this paper: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CLDQ, Chronic Liver Disease Questionnaire; HbA1c, glycosylated hemoglobin; HCV, hepatitis C virus; HRQL, health-related quality of life; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PRO, patient-reported outcome; UK, United Kingdom.

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and nonalcoholic steatohepatitis (NASH) can only be performed on liver histology, with NASH requiring the presence of lobular inflammation and hepatocyte ballooning in addition to hepatic steatosis.[3] The histologic stage of fibrosis as currently defined in a 5-tier (0–4) histologic staging in the 2 most commonly used histologic scoring systems, the NASH CRN activity score[4] and the Steatosis-Activity-Fibrosis score,[5] correlates with hepatic morbidity and overall mortality.[6] Today, NASH is the most rapidly growing cause and the second leading indication for liver transplantation in the United States.[7] Overall mortality in patients with NASH is strongly influenced by comorbidities, including abdominal obesity, arterial hypertension, insulin resistance, and dyslipidemia,[8] all of which are highly prevalent in real-world cohorts, in particular in patients with advanced fibrosis.[9]

Patients with chronic liver disease exhibit nonspecific symptoms but commonly report fatigue and abdominal discomfort. These symptoms can add to the disease burden and lead to a significant impairment in the quality of life.[10] In chronic hepatitis C, it has been proposed that these effects add to the economic burden of the disease by increasing leave time from work and loss in productivity.[11] Previous studies in patients with NAFLD observed an association between fatigue and daytime sleepiness with the degree of insulin resistance but not with the histologic disease severity.[12] In a U.S. population, NAFLD and NASH caused an incremental decrease of physical health scores, but no association of NASH or mental health scores with the degree of fibrosis was reported.[13] With the emergence of medical therapy for NASH, it will be of importance to identify patients with the highest unmet need for treatment. Patient-reported outcomes (PROs) are an important tool to assess the individual burden of a disease. Different tools have been developed to assess health-related quality of life (HRQL). The Chronic Liver Disease Questionnaire (CLDQ) is a liver disease–specific, multidimensional concept, which evaluates emotional, mental, physical, and social functioning categories.[14] Therefore, it more specifically addresses symptoms of patients with chronic liver disease, including extrahepatic manifestations, compared with traditional HRQL measures such as the SF-36 health survey questionnaire.[15–17] Lower CLDQ scores indicate worse self-reported quality of life. Using the CLDQ, a decreased HRQL was observed in a cohort study on 150 patients with non-infectious chronic liver disease, and frequently reported symptoms included fatigue, abdominal discomfort, and anxiety.[18] In patients with hepatitis C virus (HCV) an improvement of HRQL was detectable by using the CLDQ after cure.[19] Viremia and hepatic inflammation are likewise associated with impaired HRQL in patients with chronic hepatitis B virus.[20] With ongoing refinement of the PRO instruments, the NASH CLDQ was recently introduced.[21] Beyond disease-specific aspects, HRQL can be influenced by national and social factors, but generalizability has recently been shown for other tests, suggesting that PROs can be reliably assessed and compared even between different countries.[22] The aim of this prospective study was to determine factors that affect HRQL in an European population with histologically defined NAFLD.

What You Need to Know

Background

We analyzed data from the European nonalcoholic fatty liver disease (NAFLD) registry to assess the effects of this disease on health-related quality of life (HRQL).

Findings

In an analysis of data from 304 patients with NAFLD in Europe, we found a substantial symptom burden; the mean CLDQ overall score was 5.0 $\pm$ 1.2, with the lowest scores for fatigue (4.3 $\pm$ 1.6). In addition to age, sex, and the presence of diabetes, lobular inflammation detected in liver biopsies correlated with lower HRQL.

Implications for patient care

Patients with NAFLD have lower HRQL, especially those who are older, women, or with comorbidities or more advanced disease. HRQL might be used to determine patient benefit from pharmacologic treatment or to select patients for treatment.

Materials and Methods

Patient Characteristics

Patients with NAFLD were recruited at the University Medical Centre of the Johannes Gutenberg-University, Mainz, Germany, at the Freeman Hospital Liver Unit, Newcastle Upon Tyne Hospitals NHS Trust, Newcastle upon Tyne, United Kingdom (UK), and at the University Hospital of the University of Seville, Spain, as part of the prospectively enrolling European NAFLD Registry, after written informed consent. Permission was obtained from the respective ethical commissions: Ethikkommission der Landesärztekammer Rheinland-Pfalz (Germany), the North East–Tyne & Wear South Research Ethics Committee (UK), and the Spanish authorities. Other causes of liver disease were ruled out by serologic testing; thresholds for alcoholic consumption were defined according to European Association for the Study of the Liver guidelines.[23] The prevalence of type 2 diabetes, arterial hypertension, and hyperlipidemia and the presence of metabolic syndrome were defined according to the Joint Scientific Statement for Harmonizing the Metabolic Syndrome.[24] Laboratory results included alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase, albumin, platelet count, ferritin, and glycosylated hemoglobin (HbA1c) and were obtained within 30 days of liver biopsy.

Histologic Analysis

Liver histology was assessed by central scoring from expert histopathologists who have met in person and synchronized (B.S., D.T.). NASH was diagnosed and scored according to the NASH CRN criteria.[4] Histologic scoring included hepatic steatosis grade 1, 5%–33%; 2, 33%–66%; and 3, >66% of hepatocytes affected. Also included were lobular inflammation grade 0, no inflammatory foci; grade 1, <2 foci per 200× field; grade 2, 2–4 foci per 200× field; and grade 3, >4 foci per 200× field; ballooning grade 0, no ballooned hepatocytes; grade 1, few ballooned hepatocytes; and grade 2, many/prominent ballooned hepatocytes; fibrosis stage (F) 0, no fibrosis; F1, perisinusoidal, perivenular, or portal/periportal fibrosis; F2, perisinusoidal and portal/periportal fibrosis; F3, bridging fibrosis; and F4, cirrhosis. The NAFLD activity score was calculated as the sum of the scores for steatosis (1–3), lobular inflammation (0–3), and ballooning (0–2), ranging from 1 to 8.[4]

Chronic Liver Disease Questionnaire

For HRQL the liver disease–specific questionnaire CLDQ was used in the respective language.[17,25] The CLDQ consists of 29 items on a 7-point Likert scale ranging from 1 (all of the time) to 7 (none of the time) representing the frequency of clinical symptoms and emotional problems associated with liver diseases in the last 2 weeks. It is divided into 6 subscale scores (abdominal symptoms, fatigue, systemic symptoms, activity, emotional functioning, worry) and a CLDQ overall score. By dividing each domain score by the number of items in the domain, CLDQ results can be presented on a 1–7 scale, with 1 indicating worst HRQL (bad) and 7 indicating best HRQL (good). Patients completed the questionnaire during outpatient visit within 6 months of liver biopsy. A minimal clinically important difference of 0.5 in the CLDQ was considered clinically relevant.[26]

Statistical Analysis

Descriptive statistics were computed for all variables. Spearman's rank correlation coefficient was calculated to compare lab values and CLDQ scores. Univariate regression analysis was used to examine association between 2 variables. Differences between 2 groups were calculated by Mann-Whitney *U* rank test or the Fisher exact test. The χ^2 test, respectively. Kruskal-Wallis rank test was used for multi-group comparison. Analysis of covariance was performed for multivariate testing, accounting for the confounders including country, gender, age, body mass index (BMI), and type 2 diabetes. All tests were two-tailed, with significant *P* value defined as <.05. Univariate analyses were performed by using IBM SPSS Statistic Version 21.0 (Armonk, NY.). The analysis of covariance was performed by means of SAS, Version 9.4 (SAS Institute, Cary, NC). All authors had access to the study data and had reviewed and approved the final manuscript.

Results

Patient Characteristics

A total of 304 patients were included in the study, 154 from the UK, 133 from Germany, and 17 from Spain. The mean age was 52.3 (±12.9) years, and 53.3% (n = 162) were male. The majority of patients (n = 228, 75.0%) were obese, with a median BMI of 33.3 kg/m^2 (interquartile range, 30.0–37.5). Demographic data, characteristics of liver function, histopathological features, and differences between the countries are presented in Table 1. The majority of patients had moderate steatosis (grade 2, n = 152, 51.4%), none or low-grade lobular inflammation (grade 0 or 1, n = 162, 54.7%), and none or low-grade fibrosis stage (F0-2, n = 177, 58.2%) on liver biopsy.

Differences in Health-Related Quality of Life in Europe

A comparison between the 3 enrolling European countries showed significant differences between the populations (Table 1**)**. Patients in the UK (median [range], 56 years [17–77]) and Spain (61 years [33–74]) were older compared with the entire population. Likewise, rates of obesity (total cohort vs UK, 75% vs 86%; *P* < .001) and type 2 diabetes (total cohort vs UK, 51.3% vs 61.7%; *P* < .01) were higher, whereas arterial hypertension (total cohort vs UK, 56.5% vs 66.8%; *P* < .001) was lower in the UK cohort. Interestingly, there were also significant differences in HRQL between the 3 European countries, and the UK exhibited the lowest CLDQ overall score (mean [standard deviation], 4.73 [±1.3] vs 4.99 [±1.2]; *P* < .01) (Supplementary Table 1).

Health-Related Quality of Life and Influencing Factors

Mean CLDQ overall score was 4.99 (±1.2) in the entire study population. The lowest scores were reported in the subcategory "fatigue" with a value of 4.31 (±1.6), followed by "emotional functioning" with 4.93 (±1.5). "Abdominal symptoms" and "activity" showed the highest values with 5.33 (±1.6) and 5.43 (±1.4), respectively (Table 2). Women exhibited a significantly lower CLDQ overall score than men (mean [SD], 4.62 [±1.3] vs 5.31 [±1.1]; *P* < .001). Also, all CLDQ subscale scores including abdominal symptoms, fatigue, systemic symptoms, activity, emotional functioning, and worry were significantly lower in women compared with men, with a minimal clinically important difference >0.5

Table 1. Demographic Data, Characteristics of Liver Function, Histologic Features, and Differences Between the Sub-cohorts

Parameter	Total (n = 304)	UK cohort (n = 154)	German cohort (n = 133)	Spanish cohort (n = 17)	*P* value
Male gender	162 (53.3)	87 (56.5)	69 (51.9)	6 (35.3)	.82
Age, *y* (range)	54 (17–77)	56 (17–77)	53 (21–75)	61 (33–74)	**<.05**
BMI (kg/m^2)	33.3 (30.0, 37.5)	35 (31.6, 38.7)	31.9 (28.7, 36.3)	31.2 (27.3, 37.0)	**<.001**
Obesity	228 (75.0)	133 (86.4)	85 (63.9)	10 (58.8)	**<.001**
Diabetes type 2	156 (51.3)	95 (61.7)	52 (39.1)	9 (52.9)	**<.01**
Hypertension	203 (66.8)	87 (56.5)	102 (76.7)	14 (82.4)	**<.001**
Hyperlipidemia	177 (58.2)	88 (57.1)	83 (62.4)	6 (35.3)	.07
ALT	73 (48, 110)	73 (48, 109)	81 (51 110)	33 (24, 61)	**<.01**
AST	50 (36, 69)	50 (38, 71)	51 (37, 68)	29 (24, 54)	**<.01**
γ-GT	84 (56, 162)	92 (59, 164)	80 (53, 161)	82 (45, 223)	.5
Albumin	43 (40, 45)	44 (43, 47)	41 (39, 43)	45 (43, 47)	**<.001**
Platelet count	233 (183, 283)	240 (190, 296)	226 (183, 270)	190 (176, 228)	.05
Ferritin	154 (79, 313)	130 (68, 255)	220 (117, 357)	97 (51, 155)	**<.001**
HbA1c	6.1 (5.5, 7.1)	6.3 (5.75, 7.6)	5.7 (5.3, 6.3)	6.5 (6.2, 7.4)	**<.001**
Histologic findings					
NASH	210 (69.1)	109 (70.8)	89 (66.9)	12 (70.6)	.77
Steatosis 1/2/3	100/152/44	34/79/34	58/67/7	8/6/3	**<.001**
Ballooning 0/1/2	82/163/51	44/72/31	34/81/17	4/10/3	.26
Lobular inflammation 0/1/2/3	63/162/68/3	27/68/49/2	32/87/12/1	4/7/6/0	**<.001**
Fibrosis 0/1/2/3/4	36/74/67/82/45	29/29/28/40/28	5/43/36/37/12	2/2/3/5/5	**<.001**

NOTE. Data are expressed as number (percentage) or median (25th, 75th percentiles). Histologic findings were scored according to the criteria proposed by Kleiner et al.[4] Comparisons between cohorts were carried out using the χ^2 or Kruskall-Wallis test. ALT (normal range, <50 U/L), AST (normal range, 5–35 U/L), γ-GT (normal range, 12-64 U/L), Albumin (normal range, 34–48 g/L), Platelet count (normal range, 150-450/nL), Ferritin (normal range, 20–275 ng/mL), HbA1c (normal range, 4.1%–5.6%), obesity is defined as BMI >30 kg/m^2. Boldface indicates statistical significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γ-GT, gamma-glutamyl transferase; HbA1c, glycosylated hemoglobin; NASH, nonalcoholic steatohepatitis; UK, United Kingdom.

($P < .01$) (Table 2). No correlation between CLDQ overall score and age existed (Table 3). There was a negative correlation between overall CLDQ score and obesity ($P < .001$), type 2 diabetes ($P < .001$), and dyslipidemia ($P < .01$) (Table 3). AST (r = −0.12; $P < .05$), ferritin (r = 0.166; $P < .01$), and HbA1c (r = −0.26; $P < .001$) correlated with CLDQ overall score significantly, whereas there was no correlation regarding ALT (r = 0.04) or gamma-glutamyl transferase (r = −0.08) (Table 3). With regard to the subscale scores, fatigue scored the lowest compared in all countries (Supplementary Table 1).

Impact of Histologic Features of Nonalcoholic Fatty Liver Disease on Health-Related Quality of Life

NASH was present in 210 patients (69.1%), with no influence of gender. Obesity (54.9% vs 20.1%; $P < .05$) and type 2 diabetes (39.9% vs 11.5%; $P < .01$) were more prevalent in NASH compared with NAFL, whereas age, hypertension, dyslipidemia, and hyperferritinemia were not different. AST ($P < .001$), ALT ($P < .05$), and HbA1c ($P < .001$) were significantly higher in NASH compared with NAFL. NASH was associated with a significantly lower HRQL compared with patients with NAFL (mean [standard deviation], 4.85 [±1.3] vs 5.31 [±1.1]; $P < .01$). In addition, patients with NASH scored significantly lower on all CLDQ subscales except for "abdominal symptoms" and "emotional function" (Table 4). By using a minimal clinically important difference threshold of 0.5, the subscales "fatigue" and "systemic symptoms" reached clinically meaningful differences, whereas CLDQ overall score (Δ 0.46) showed a clear trend toward impaired HRQL in NASH.

The histologic features of NAFLD on liver biopsy had a significant impact on HRQL. Patients with more severe hepatic steatosis exhibited a lower HRQL score (grade 3 vs 1, 4.5 [±1.4] vs 5.3 [±1.1]; $P < .05$). Similarly, more severe ballooning (grade 2 vs 0, 4.7 [±1.3] vs 5.3 [±1.2];

Table 2. Differences in Health-Related Quality of Life Concerning Gender Aspects

Parameter	Total (n = 304)	Male (n = 162)	Female (n = 142)	*P* value
CLDQ overall score	4.99 (±1.2)	5.31 (±1.1)	4.62 (±1.3)	**<.001**
Abdominal symptoms	5.33 (±1.6)	5.69 (±1.4)	4.92 (±1.7)	**<.001**
Fatigue	4.31 (±1.6)	4.61 (±1.5)	3.96 (±1.5)	**<.001**
Systemic symptoms	5.09 (±1.3)	5.43 (±1.2)	4.71 (±1.3)	**<.001**
Activity	5.43 (±1.4)	5.79 (±1.3)	5.02 (±1.4)	**<.001**
Emotional functioning	4.93 (±1.5)	5.27 (±1.4)	4.54 (±1.5)	**<.001**
Worry	5.18 (±1.5)	5.45 (±1.3)	4.86 (±1.6)	**<.01**

NOTE. Data are expressed as means and standard deviations. Comparisons between groups were carried out using the Mann-Whitney *U* test. Boldface indicates statistical significance.
CLDQ, Chronic Liver Disease Questionnaire.

Table 3. CLDQ in Relation to Presence or Absence of Patient Characteristics and Laboratory Results

Characteristics	CLDQ overall score: Characteristic present	CLDQ overall score: Characteristic absent	*P* value
Age ≥54 y	4.94 (±1.2)	5.05 (±1.3)	.37
Obesity (BMI >30 kg/m^2)	4.83 (±1.2)	5.46 (±1.1)	**<.001**
Diabetes type 2	4.74 (±1.2)	5.25 (±1.2)	**<.001**
Hypertension	4.97 (±1.2)	5.04 (±1.3)	.51
Hyperlipidemia	4.84 (±1.2)	5.24 (±1.2)	**<.01**

	Correlation coefficient (r) with CLDQ score	*P* value
ALT	0.04	.53
AST	–0.12	**.04**
γ-GT	–0.08	.16
Albumin	<0.01	.97
Platelet count	–0.12	.05
Ferritin	0.17	**<.01**
HbA1c	–0.26	**<.001**

NOTE. Data presented as means and standard deviations. Obesity is defined as BMI >30 kg/m^2. Statistical dependence between parameters of metabolic syndrome and CLDQ was measured by Mann-Whitney *U* test; to compare laboratory levels and CLDQ score, Spearman's rank correlation coefficient was performed. Boldface indicates statistical significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLDQ, Chronic Liver Disease Questionnaire; γ-GT, γ-glutamyl transferase; HbA1c, glycosylated hemoglobin.

$P < .05$) and severe lobular inflammation (grade 3 vs 0, 3.9 [±1.8] vs 5.3 [±1.2]; $P < .001$), all with a difference of >0.5 points, were associated with lower HRQL. Advanced fibrosis and compensated cirrhosis (F3/F4) were observed in 127 patients (41.8%), and these exhibited a trend toward lower HRQL (F3-4 vs F0-2, 4.9 [±1.2] vs 5.1 [±1.3]; $P = .07$). In contrast to the histologic features of steatohepatitis, this was not statistically significant or clinically meaningful. Figure 1 summarizes the histologic features and the associated CLDQ overall scores. On multivariate analysis, correcting for country, gender, age, BMI, and type 2 diabetes, an independent association between impaired HRQL and hepatic inflammation ($P < .05$) but not fibrosis ($P = .47$) was detected. Also, gender ($P < .0001$), age ($P < .05$), BMI ($P < .001$), and type 2 diabetes ($P < .01$) were independently associated with impaired HRQL (Supplementary Table 2).

Table 4. Comparison of Health-Related Quality of Life in NAFL and NASH

Parameter	Total (n = 304)	NAFL (n = 94)	NASH (n = 210)	*P* value
CLDQ overall score	4.99 (±1.2)	5.31 (±1.1)	4.85 (±1.3)	**<.01**
Abdominal symptoms	5.33 (±1.6)	5.64 (±1.3)	5.19 (±1.7)	.088
Fatigue	4.31 (±1.6)	4.76 (±1.5)	4.10 (±1.6)	**<.01**
Systemic symptoms	5.09 (±1.3)	5.45 (±1.2)	4.93 (±1.4)	**<.01**
Activity	5.43 (±1.4)	5.74 (±1.3)	5.29 (±1.4)	**<.01**
Emotional functioning	4.93 (±1.5)	5.15 (±1.5)	4.83 (±1.5)	.067
Worry	5.18 (±1.5)	5.47 (±1.5)	5.04 (±1.5)	**<.05**

NOTE. Data are expressed as means and standard deviations. Comparisons between groups were carried out using the Mann-Whitney *U* test. Boldface indicates statistical significance.
CLDQ, Chronic Liver Disease Questionnaire; NAFL, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Discussion

The current study explored HRQL in patients with biopsy proven NAFLD from 3 European centers. HRQL is an important facet when assessing the burden of a chronic disease. Despite the lack of specific symptoms in liver disease, patients can experience impairment in the quality of life at an individual level.[27] In patients with NAFLD and other chronic liver disease, fatigue and impaired sleeping quality are the most frequently reported findings.[12,27,28] Likewise, the number of comorbidities and medications are negatively correlated with HRQL in patients with chronic liver disease.[18] The striking finding of the current analysis in this well-characterized European cohort was that, in contrast to the published data on predictors of overall and liver-specific mortality, lobular inflammation correlated independently with HRQL.[6,29] These results differ from the NASH CRN cohort, which found lower HRQL using the generic short form-36 (SF-36) in NASH compared with a healthy U.S. population and a significant effect in cirrhosis only.[13] The apparent divergence of fibrosis on mortality and HRQL is intriguing and potentially reflects differences in the underlying mechanisms that contribute to progression of the respective histologic lesion and the loss in HRQL. Metabolic inflammation creates a milieu in which liver cell injury and fibrogenesis occur and drive disease progression over years. Various studies have identified hepatic fibrosis but not inflammation or steatosis on liver biopsy as the histologic feature that correlates best with overall and liver-related mortality.[6,29] Although inflammation and steatosis are prerequisites for the diagnosis and disease progression, these features are more dynamic compared with hepatic fibrosis. On the other hand, hepatic fibrosis reflects an aggregate of liver injury that builds up over time and can be detected on liver biopsy despite sampling variability. Nonetheless, the disease activity, namely inflammation and ballooning, has been linked to elevated cytokine levels and markers of systemic inflammation.[30] These inflammatory markers and metabolic stress are known to negatively affect the mood and promote depressive symptoms.[31]

Data from clinical trials in chronic HCV or hepatitis B virus infection support a dominant role of inflammation on HRQL. Viral elimination or suppression after antiviral therapy was associated with improved HRQL, which argues for an effect of inflammation on PROs, whereas improvement of fibrosis did not affect HRQL.[20,26,32] Also, improvement of HRQL was comparable in patients with

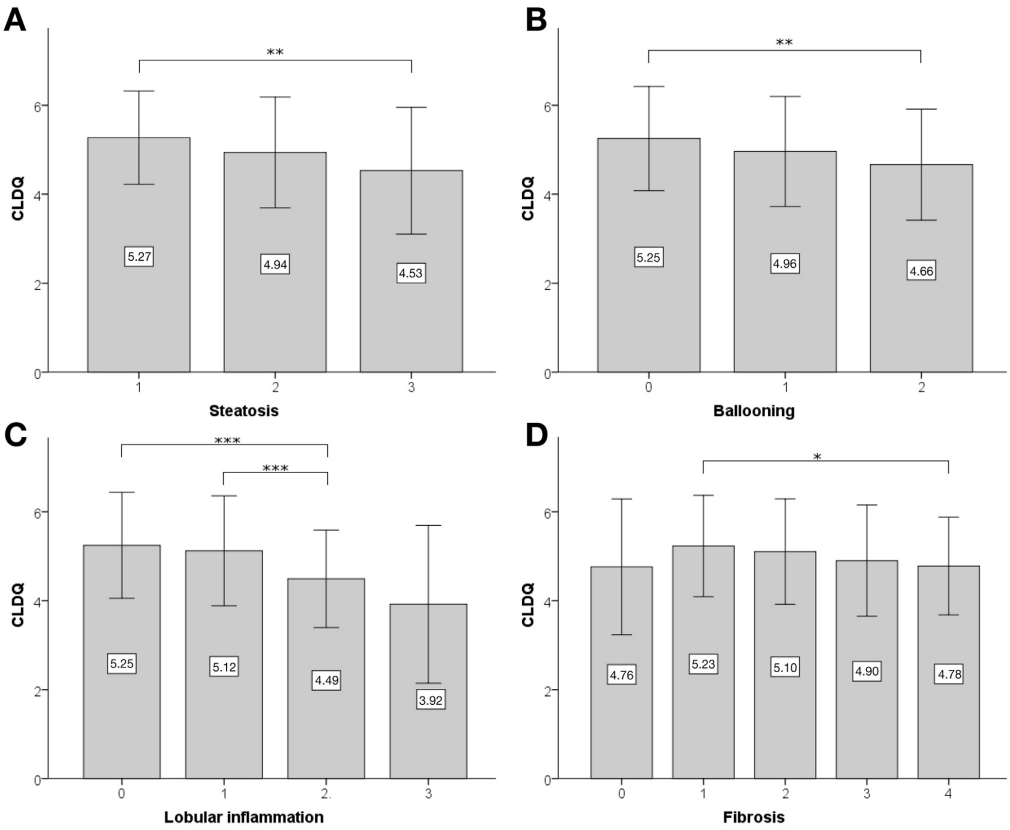

Figure 1. Impact of histologic features of nonalcoholic fatty liver disease on health-related quality of life. (*A*) Steatosis, (*B*) ballooning, (*C*) lobular inflammation, (*D*) fibrosis. CLDQ, Chronic Liver Disease Questionnaire.

early and advanced fibrosis after cure using direct-acting antivirals.[26] In a recent trial in patients with histologically confirmed NASH and fibrosis stage 2 or 3, an improvement of fibrosis by at least 1 stage resulted in an improvement in HRQL.[33] Beyond histologic findings, a significant negative impact of metabolic comorbidities, including type 2 diabetes, obesity, or dyslipidemia, on HRQL was observed. In line with the published data, fatigue was the most frequently reported symptom.[12,27,34]

The burden of disease for NAFLD is high, and an exponential increase in Europe is predicted in the next few years.[35] In Germany, France, Italy, and UK there are approximately 52 million people living with NAFLD, and the connected annual costs have been estimated at 35 billion euros. These costs arise from liver-related morbidity and associated comorbidities that amount to spending in health care but also indirect cost related to lost work productivity.[36]

The current analysis highlights the impact of lobular inflammation on HRQL, which to a lower extent translated into differences in HRQL between NAFL and NASH. Currently, clinical trials are being conducted to assess the resolution of steatohepatitis and improvement or stabilization of hepatic fibrosis as a primary endpoint.[37] On the basis of the current analysis it can be expected that improvement of steatohepatitis, and in particular lobular inflammation, will have measurable influence on HRQL even independently of fibrosis improvement. Clinically meaningful differences were also detected with regard to gender. Women scored lower in all subcategories of the CLDQ across all countries, indicating that the burden of disease in women could be higher. This effect was not explained by disease activity or advanced stage. Interestingly, these findings are replicated in studies on HCV and human immunodeficiency virus co-infected patients that also showed significantly lower HRQL in women.[19] Thus, it seems plausible that

CLDQ has a higher sensitivity to detect impairment in the quality of life in women compared with men. Future tools of HRQL will have to account for this gender-specific difference.

The CLDQ assesses not only symptoms but also social and emotional factors at a superficial level by using 4–5 questions in the respective subsections. Therefore, it has proven particularly feasible in an outpatient setting with limited time resources. The CLDQ represents a disease-specific tool with the capability to detect subtle disease-specific aspects that are missed by more commonly used generic tools. Nonetheless, the ability of the CLDQ to differentiate subtler aspects can be questioned because most patients scored within a range of 2.5 points on this 7-point Likert scale, and further refinements are now available.[38] Beyond the assessment of treatment response, HRQL could be potentially useful in prioritizing patients for lifestyle interventions or pharmacologic therapies in the future.

In summary, the current study highlights the link of impaired HRQL with liver parenchymal inflammation in patients with NAFLD from Northern, Middle, and Southern Europe. These findings contradict frequent perception that patients with chronic liver disease are asymptomatic. Our findings underline the need for an appropriate tool to assess the symptoms that contribute to the high disease burden in NASH. Because NAFLD is a highly prevalent disease that causes a distinct loss in HRQL and eventually also poses an economic burden, a high priority should be placed on prevention and treatment. With the emergence of medical therapy, the improvement in HRQL will likely influence the choice of drug in the future.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2018.12.016.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Table 1. Comparison of Health-Related Quality of Life in Sub-cohorts

Parameter	Total (n = 304)	UK cohort (n = 154)	German cohort (n = 133)	Spanish cohort (n = 17)	*P* value
CLDQ overall score	4.99 (±1.2)	4.73 (±1.3)	5.27 (±1.1)	5.14 (±1.1)	**<.01**
Abdominal symptoms	5.33 (±1.6)	5.24 (±1.6)	5.51 (±1.5)	4.76 (±1.6)	.12
Fatigue	4.31 (±1.6)	4.12 (±1.6)	4.48 (±1.5)	4.64 (±1.7)	.09
Systemic symptoms	5.09 (±1.3)	4.82 (±1.4)	5.37 (±1.2)	5.35 (±1.2)	**<.01**
Activity	5.43 (±1.4)	5.21 (±1.5)	5.73 (±1.2)	5.12 (±1.4)	**<.01**
Emotional functioning	4.93 (±1.5)	4.57 (±1.6)	5.30 (±1.3)	5.32 (±1.4)	**<.001**
Worry	5.18 (±1.5)	4.91 (±1.7)	5.46 (±1.3)	5.38 (±1.1)	**<.01**

NOTE. Data are expressed as means and standard deviations. Comparisons between cohorts were carried out using the Kruskal-Wallis test.
CLDQ, Chronic Liver Disease Questionnaire; UK, United Kingdom.

Supplementary Table 2. Associations Between Impaired HRQL and Different Parameters From Analysis of Covariance

Parameter	DF	*P* value (F test, analysis of covariance)
Country	2	.13
Gender	1	**<.0001**
Age	1	**.037**
BMI	1	**.0003**
Type 2 diabetes	1	**.004**
Steatosis	2	.22
Ballooning	2	.49
Inflammation	3	**.038**
Fibrosis	1	.47

NOTE. Analysis of covariance after correction for confounders including country, gender, age, BMI, and type 2 diabetes.
BMI, body mass index; DF, degrees of freedom.

Appendix B: Performance of the PROC3 collagen neo-epitope biomarker in non-alcoholic fatty liver disease

Research Article JHEP|Reports

Performance of the PRO-C3 collagen neo-epitope biomarker in non-alcoholic fatty liver disease

Authors

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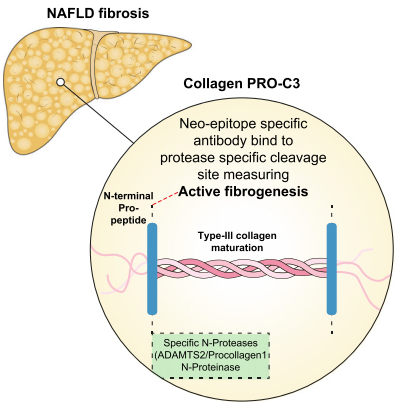
Graphical abstract



Biomarker panel development for the detection of advanced fibrosis

PRO-C3 biomarker **diagnostic** context of use

- A large international cohort of biopsy confirmed NAFLD patients
- Majority of biopsies centrally read (high kappa value inter-observer agreement)
- Analysis of biomarker performance in the current study has been conducted by researchers fully independent of the biomarker manufacturer

PRO-C3 platelets + **Age BMI T2DM**

ABC3D
(Age >50 = 1, BMI >30 = 1, Platelet count <200, Pro-C3 >15.5 = 1, T2DM = 2
Score >3

FIBC3
FIBC3 score >-0.4

	FIBC3	FIB4	ABC3D
AUROC	0.83	0.76	0.81
Sensitivity	75.00	21.00	66.00
Specificity	75.00	94.00	75.00

Highlights

- Plasma PRO-C3 levels correlate with severity of steatohepatitis and fibrosis stage.
- FIBC3 panel achieves good sensitivity and specificity for the identification of F≥3 fibrosis in NAFLD.
- FIBC3 panel uses a single threshold value, eliminating indeterminate results and outperforming other non-invasive tools.
- A simplified version (ABC3D) is readily amenable to use in clinical practice.

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Lay summary

We performed a comprehensive, independent evaluation of a collagen biomarker (PRO-C3) to detect and quantify liver fibrosis in patients with non-alcoholic fatty liver disease (NAFLD). We report the development of 2 diagnostic panels using PRO-C3 to identify patients with advanced fibrosis, one optimal but more complex to calculate (FIBC3), the other easier to use (ABC3D) whilst still performing well.

stepwise multiple logistic regression analysis to identify independent factors associated with fibrosis. Variables with p <0.05 by multivariate analysis were used to construct scoring systems (FIBC3 and ABC3D) to predict advanced fibrosis. Optimal cut-offs for each component of ABC3D were selected using the Youden index (J-Index) which attributes equal value to sensitivity and specify. Cross-validation was performed using the leave-one-out method to facilitate the calculation of over-fit bias reduced estimates. We calculated reduced bias estimates of predicted probability. This involved removing each individual subject and re-estimating the model parameters and then classifying the subject based on the new parameters. This enabled us to interrogate a suspicious positive or negative validation subject.

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ALT (U/L)	69 ± 41	66 ± 39	71 ± 42	0.166
High ALT (>40 U/L)	340 (76%)	112 (74%)	228 (77%)	0.585
AST (U/L)	47 ± 26	47 ± 26	48 ± 26	0.339
Albumin (g/dl)	44 ± 5	44 ± 4	44 ± 5	0.780
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features on biopsy. The current study addresses the performance of the PRO-C3 biomarker within the FDA BEST (Biomarkers, EndpointS and other Tools) defined *diagnostic* context of use.[11]

Blood-based non-invasive tests for fibrosis can be dichotomised into "indirect makers", including simple non-invasive fibrosis scores derived from clinical and biochemical indices, such as the fibrosis-4 (FIB4) score and the NAFLD fibrosis score (NFS),[12–16] and "direct biomarkers" that measure collagen deposition or matrix turnover.[17,18] The majority of non-invasive tests exhibit high negative predictive value, implying that they are best employed to exclude patients without advanced fibrosis (Kleiner ≤F2). However, many issues exist with currently available biomarkers. For example, FIB4 and NFS provide "indeterminate" results in a quarter of patients[19] and although elastography based techniques such as Fibroscan™ (vibration controlled transient elastography [VCTE]) have a competitive diagnostic accuracy, they require specialist equipment, are operator dependent and exhibit low success rates in obese patients.[20] Magnetic resonance elastography can accurately diagnose fibrosis in patients with NAFLD.[21,22] However, it is expensive and not widely available in most centres. A mandate therefore exists for improved biomarkers.

Research exploiting knowledge of collagen structure and protease-protein interactions have resulted in the design of a specific ELISA that measures ADAMS2 mediated collagen cleavage during the formation of type III collagen in fibrogenesis.[23,24] Previous studies have shown that measuring formation of type III collagen neo-epitopes (PRO-C3) as a single diagnostic marker or by incorporation into a diagnostic panel can provide a reasonably accurate assessment of disease stage and activity, but to date the diagnostic panels require complex mathematical calculations necessitating the use of an online App.[25–30] Similarly, NFS and FIB4 require the use of online calculators to generate a result. This may be onerous in a busy clinical environment, limiting adoption in the primary care setting.[31,32] A simplified but accurate fibrosis assessment algorithm would therefore help physicians to risk stratify patients without recourse to an online calculator.

In the current study, we seek to: i) assess the performance of PRO-C3 as a NASH-fibrosis biomarker within the BEST *diagnostic* context of use; ii) develop and validate a novel biomarker panel incorporating PRO-C3 and determine its performance in comparison to established clinical scores and previously reported biomarker panels; and iii) develop and validate a simplified clinical tool that is both accurate and clinically accessible immediately.

Materials and methods

Study design and participants

Fig. 1 shows the flow of patients through the study. Participants were recruited at 7 specialist European centres. Patients eligible for inclusion were ≥18 years, with suspected NAFLD undergoing a diagnostic liver biopsy on clinical grounds. Patients were excluded if they had evidence of coexistent liver disease or consumed greater than 30 g of alcohol per day for males or greater than 20 g per day for females. The human biological samples were sourced ethically following receipt of informed consent from each patient and their research use was in accordance with the terms of the informed consents under an IRB/EC approved protocol at participating centres.

Clinical and laboratory assessments

Gender, age and body mass index (BMI; weight (kg)/height (m^2)) were recorded for all patients at time of index liver biopsy. Patients were classified as having type 2 diabetes mellitus (T2DM) if HbA1c

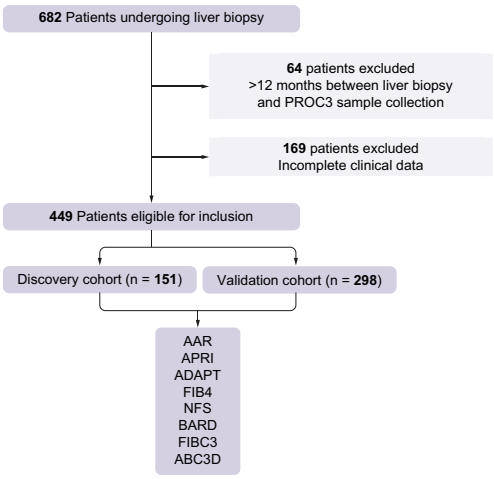

Fig. 1. Patient flow for analysis inclusion.

was >6.5% or they were receiving dietary, oral hypoglycaemic drug or insulin treatment for T2DM. Blood tests taken at the time of liver biopsy were used to calculate the simple non-invasive scores. The FIB4 score, APRI (aspartate aminotransferase to platelet ratio index), NFS, ADAPT (Age, Diabetes, PRO-C3 and platelets panel) score and BARD (BMI, aspartate aminotransferase to alanine aminotransferase ratio [AAR], T2DM) score were calculated and applied as previously described.[13,29,33–35] PRO-C3 and additional biomarkers PRO-C6, PRO-C4, C4M were assessed using competitive ELISAs (Nordic Bioscience A/S, Denmark) measured by experienced technicians unaware of any associated clinical data.[23,36]

Histological assessment

Liver biopsies were performed at each centre as per unit protocol. Target biopsy length was ≥15 mm. Biopsies were stained with haematoxylin and eosin and Masson's trichrome. Histological diagnosis, grade of steatosis and scoring for NAFLD activity and fibrosis stage were performed by expert liver pathologists at each study site according to the NASH Clinical Research Network (CRN) classification.[37] To reduce the element of inter-observer variability, over half of all biopsies (254, 57%) in our study were centrally reviewed by an expert member of the Elucidating Pathways of Steatohepatitis (EPoS) Histopathology Group (DT). A weighted kappa coefficient of 0.90 for fibrosis stage was established, demonstrating a very high level of inter-observer agreement.

Statistical analysis

The primary endpoint of the study was to predict the presence of advanced fibrosis (stages 3–4). The combined cohort of 449 patients was randomly separated into approximately 1/3 (n = 151) (discovery cohort) and 2/3 (n = 298) of patients (validation cohort) for model building and validation. Continuous variables were compared using the *t* test and categorical variables using Fisher's exact test. The Kruskal-Wallis test was used to perform comparisons between mean marker levels followed by Dunn's multiple comparison tests. In the discovery cohort, significant variables on univariate analysis (p <0.05) were included in the backward

stepwise multiple logistic regression analysis to identify independent factors associated with fibrosis. Variables with p <0.05 by multivariate analysis were used to construct scoring systems (FIBC3 and ABC3D) to predict advanced fibrosis. Optimal cut-offs for each component of ABC3D were selected using the Youden index (J-Index) which attributes equal value to sensitivity and specify. Cross-validation was performed using the leave-one-out method to facilitate the calculation of over-fit bias reduced estimates. We calculated reduced bias estimates of predicted probability. This involved removing each individual subject and re-estimating the model parameters and then classifying the subject based on the new parameters. This enabled us to interrogate a suspicious positive or negative validation subject.

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Mann-Whitney/ t tests were used to test for significant differences within continuous variables and Chi-Square test was used for categorical variables.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; T2DM, type 2 diabetes mellitus.
^The table shows the mean ± SD for continuous variables, number (%) for binary variables, and number per group for categorical variables.

for all 2-way interactions with no significant outcomes (p >0.05). These 5 variables were incorporated into a model that distinguished advanced fibrosis (F3-4) from mild fibrosis (F0-F2). The diagnostic panel "FIBC3" was calculated from the regression formula for prediction of severity of fibrosis: -5.939 + (0.053*Age) + (0.076*BMI) + (1.614*T2DM) – (0.009*platelets) + (0.071*PRO-C3). FIBC3 correlated strongly with fibrosis stage (rho = 0.630, p <0.0001), which remained significant independently of NAS. In the discovery cohort, the AUROC for FIBC3 was 0.89 (95% CI 0.843–0.941, p <0.001).

To facilitate adoption in a clinical setting, a simplified score based on the same 5 variables identified as significant on univariate analysis and weighted according to their odds ratio (OR) values was generated. The derived "ABC3D" score comprises: **A** = **A**ge>50 years, **B** = **B**MI>30, **C** = platelet **C**ount<200, **3** = PRO-C3>15.5 ng/ml, **D**iabetes = present. Optimal thresholds for each variable were selected by maximising the Youden index for the corresponding ROC curves. The presence of each factor scored 1 point, except for T2DM which, with an OR of 5, was awarded 2 points to yield a maximum score of 6. In the discovery cohort, the AUROC for ABC3D was 0.88 (95% CI 0.822–0.929, p <0.001).

Validation of FIBC3 and ABC3D model accuracy and derivation of diagnostic thresholds for advanced fibrosis

The diagnostic accuracy of these models for the detection of advanced fibrosis was confirmed in a the validation cohort (n = 298) and also in the overall combined cohort (n = 449). Diagnostic accuracy was assessed by the standard AUROC and also the weighted AUROC computed using the Obuchowski measure to account for spectrum effect and ordinal scale.

Table 3. Diagnostic accuracy of non-invasive tests by detecting Histologic stage F3–F4 and weighted AUROC derived from the Obuchowski measure.

			Combined cohort (n = 449)		
Non-invasive test	**AUROC**	**95% CI**	**Adj AUROC**	**SD**	**95% CI**
AAR	0.67	0.615–0.716	0.62	0.019	0.581–0.653
APRI	0.75	0.698–0.794	0.68	0.017	0.652–0.717
BARD	0.71	0.664–0.761	0.67	0.017	0.642–0.707
FIB4	0.78	0.732–0.820	0.70	0.015	0.671–0.731
NFS	0.79	0.751–0.838	0.72	0.015	0.694–0.752
ADAPT	0.85	0.815–0.888	0.77	0.014	0.739–0.794
PRO–C3	0.76	0.718–0.811	0.69	0.017	0.660–0.726
FIB–C3	0.85	0.812–0.886	0.77	0.013	0.745–0.797
ABC3D	0.83	0.793–0.868	0.76	0.013	0.730–0.783
***p* value**	<0.0001				
			Discovery cohort (n = 151)		
AAR	0.66	0.579–0.751	0.62	0.031	0.555–0.675
APRI	0.75	0.669–0.830	0.69	0.028	0.638–0.748
BARD	0.76	0.683–0.834	0.69	0.028	0.637–0.746
FIB4	0.80	0.726–0.867	0.70	0.026	0.651–0.751
NFS	0.85	0.791–0.911	0.71	0.023	0.669–0.758
ADAPT	0.86	0.800–0.917	0.74	0.025	0.695–0.793
PRO–C3	0.75	0.661–0.831	0.68	0.031	0.617–0.740
FIB–C3	0.89	0.843–0.941	0.75	0.021	0.707–0.789
ABC3D	0.88	0.822–0.929	0.75	0.022	0.704–0.790
***p* value**	<0.0001				
			Validation cohort (n = 298)		
AAR	0.66	0.599–0.725	0.62	0.024	0.571–0.663
APRI	0.75	0.686–0.805	0.68	0.021	0.640–0.722
BARD	0.69	0.624–0.749	0.66	0.021	0.623–0.705
FIB4	0.76	0.707–0.819	0.70	0.019	0.644–0.739
NFS	0.76	0.701–0.818	0.73	0.019	0.692–0.766
ADAPT	0.85	0.803–0.896	0.78	0.017	0.749–0.815
PRO–C3	0.78	0.727–0.838	0.70	0.020	0.622–0.741
FIB–C3	0.83	0.777–0.880	0.79	0.017	0.753–0.819
ABC3D	0.81	0.755–0.856	0.76	0.017	0.730–0.795
***p* value**	<0.0001				

*Prevalence advanced fibrosis *combined cohort = 0.37 *Discovery cohort = 0.40 * Validation cohort = 0.36
*DeLong DeLong Clarke test for comparison of AUROC

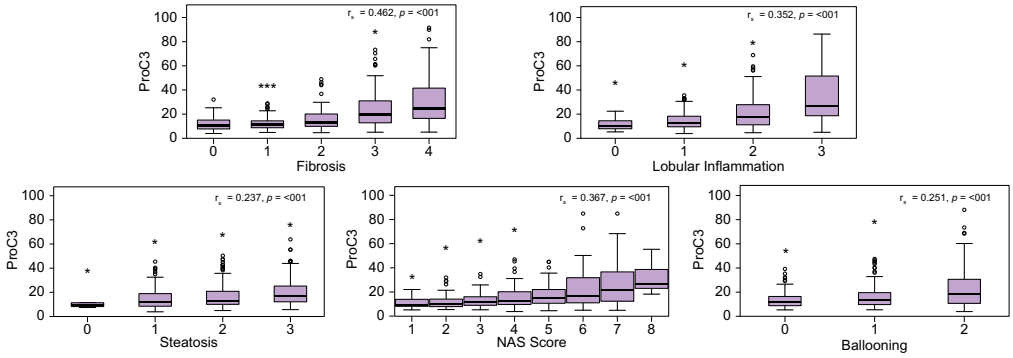

Fig. 2. PRO-C3 and its association with non-alcoholic fatty liver disease severity (complete cohort n = 449). Spearman's correlation coefficient r_s measures the strength and direction of association between 2 variables. Independent samples were compared using the Kruskal-Wallis test. All data are represented as medians, with variation in expression shown in Tukey plots. *P* values <0.05 were considered significant.

PRO-C3 levels correlated with steatohepatitis and fibrosis stage

Across all histological features (steatosis, lobular inflammation, hepatocyte ballooning, fibrosis), PRO-C3 was positively associated with increasing NAFLD severity (Fig. 2). In the discovery cohort (n = 151), PRO-C3 correlated with the NAFLD activity score (NAS) (rho = 0.304, p <0.0001) and fibrosis stage (rho = 0.422, p <0.0001). Confirming that PRO-C3 is primarily a fibrosis marker, the correlation with fibrosis stage remained significant when controlling for NAS however the converse did not hold true. Indeed, PRO-C3 exhibited the strongest correlation with fibrosis stage when compared to a number of other putative extracellular matrix turnover biomarkers (PRO-C6 (rho = 0.355), PRO-C4 (rho = 0.279), C4M (rho = 0.177), p <0.05).

In the discovery cohort (n = 151) an optimal PRO-C3 cut-off level for the detection of advanced fibrosis was determined. PRO-C3 >15.5 ng/ml had an AUROC of 0.73 for the detection of advanced fibrosis ≥F3 (sensitivity 60%, specificity 74%, accuracy 68%). This was replicated in the validation cohort (n = 298) (AUROC = 0.78, sensitivity 72%, specificity 71%, accuracy 71%) (Table S2). The sensitivity and specificity for fibrosis across a range of PRO-C3 thresholds are reported for the overall cohort (Table S3).

Development of panels incorporating PRO-C3 that are diagnostic for advanced fibrosis

To identify other clinical factors that readily predict the presence of fibrosis, additional analyses were conducted. Table 2 shows the results of univariate and multivariate analyses preformed in the discovery cohort. Using backward logistic regression, 5 variables remained significantly associated with advanced fibrosis: age, BMI, T2DM, platelets and PRO-C3. No multi-collinearity was identified between variables used in the model. Variables were assessed

Table 2. Variables Associated with the Presence of Advanced Fibrosis (stage F3-4) in the Discovery Cohort (n = 151).

	Univariate			Adjusted (Multivariate)		
Variable	**Odds Ratio**	**95% CI**	***p* value**	**Odds Ratio**	**95% CI**	***p* value**
Age	1.088	1.049–1.128	<0.0001	1.055	1.008–1.103	0.022
Gender	1.172	0.599–2.291	0.643			
BMI	1.090	1.035–1.148	0.001	1.079	1.014–1.148	0.017
T2DM	8.570	4.003–18.348	<0.0001	5.023	1.920–13.140	0.001
ALT	1.002	0.994–1.011	0.611			
AST	1.020	1.005–1.034	0.007			
Albumin	0.934	0.853–1.021	0.133			
Platelets	0.986	0.986–0.979	<0.0001	0.991	0.982–1.000	0.039
Cholesterol	0.841	0.714–0.990	0.038			
Triglycerides	1.024	0.952–1.101	0.520			
PRO-C3	1.079	1.039–1.120	<0.0001	1.074	1.023–1.127	0.004
AST-ALT Ratio	3.072	1.119–8.436	0.029			
FIBC3: –5.939 + (0.053*Age) + (0.076*BMI) + (1.614*T2DM) – (0.009*platelets) + (0.071*PRO–C3) **ABC3D:** **A**ge >50 = 1 point, **B**MI >30 = 1 point, platelet **C**ount <200 = 1 point**,** PRO–C**3** >15.5 = 1 point, **D**iabetes = 2 points						

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; T2DM, type 2 diabetes mellitus.

Table 4. Optimal cut-off values for the detection of advanced fibrosis (≥F3) as per Youden index derived in discovery cohort (prevalence 0.40, n = 151) and applied in validation cohort (prevalence 0.36, n = 298).

Panel	AUC	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
FIB-C3	0.89	>–0.4	83	80	74	88	81
ABC3D	0.88	>3	77	82	74	84	80
			Validation cohort				
		Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
AAR		>0.8	46	71	47	70	62
APRI		>1.5	11	96	63	66	66
BARD		≥2	76	51	47	79	60
FIB4		>2.67	21	94	67	68	68
NFS		>0.676	27	95	78	70	71
ADAPT		>6.3	76	75	63	86	76
FIB–C3		>–0.4	75	75	62	84	75
ABC3D		>3	66	75	61	80	73

PPV, positive predictive value; NPV, negative predictive value.

For FIBC3, the AUROC remained high in both the validation cohort (0.83, 95% CI 0.777-0.880) and the combined cohort (0.85, 95% CI 0.812-0.886). The weighted AUROC was calculated to be 0.77, 0.75 and 0.79 in the combined, discovery and validation cohorts, respectively. Similar results were obtained for ABC3D with AUROC of 0.81 and 0.83 in the validation and combined cohorts, respectivelY (Table 3). Reduced bias estimates of predicted probability were calculated in the discovery and validation cohorts, employing the leave-one-out method of cross-validation as previously described. To assess the added value of including PRO-C3 in the diagnostic model, we removed PRO-C3 from the FIBC3 model. This yeilded AUROCs of (0.80, 0.86 and 0.76) in the total, discovery and validation cohorts, respectively. These improved to (0.85, 0.89 and 0.83) with the inclusion of PRO-C3 in the model.

An optimal FIBC3 threshold value of >-0.4 was chosen using the Youden index (sensitivity 83%, specificity 80%, positive predictive value [PPV] 74% and negative predictive value [NPV] 88%). An optimal ABC3D cut-off level for the detection of advanced fibrosis was >3. In the validation cohort (n = 298), FIBC3 exhibited a sensitivity of 75%, specificity of 75%, accuracy of 75% (Table 4). In the discovery cohort, ABC3D exhibited a sensitivity of 77%, specificity of 82%, and accuracy of 80%. This was replicated in the validation cohort, where a sensitivity of 66%, specificity of 75% and accuracy of 73% were observed.

Both FIBC3 and ABC3D performance were superior to simple non-invasive scores in common use, with accuracies of 75% and 73%, respectively. Performance characteristics of FIBC3 and the simplified ABC3D score were comparable to the recently described ADAPT score (Table 4). Comparing AUROCs using the DeLong, DeLong and Clarke-Pearson method confirmed that FIBC3 and ABC3D have similar performance characteristics (p = 0.1422) as do FIBC3 and ADAPT (p = 0.1859). Using the FIBC3 model, the optimal threshold correctly staged 224 out of 298 patients (75%) in the validation cohort, compared to 227 patients (76%) with ADAPT and 217 (73%) with ABC3D. Considering NPV, of 191 patients with mild fibrosis, 144 (75%) were staged correctly using FIBC3 or ABC3D, equal to ADAPT (75%) (Table 5). In the combined cohort (n = 449), 347 of the patients (77%) were correctly staged using FIBC3, which outperformed both FIB4 at 304 (68%) and ADAPT at 341 (76%). The most simple model, ABC3D, had a diagnostic accuracy of 75% correctly classifying 338 cases into mild or severe fibrosis.

Table 5. Validation cohort divided into mild and severe fibrosis (prevalence 0.39, n = 298).

	F0–2 'Rule out' advanced fibrosis				F3–4 'Rule in' advanced in severe		
	Correctly identified	Indeterminate	Incorrectly identified		Correctly identified	Indeterminate	Incorrectly identified
N = 191	n/N (%)	n/N (%)	n/N (%)	N = 107	n/N (%)	n/N (%)	n/N (%)
AAR <0.8	135/191 (71)		56/191 (29)	AAR >0.8	49/107 (46)		58/107 (54)
APRI <0.5	112/191 (59)	72/191 (38)	7/191 (3)	APRI >1.5	12/107 (11)	72/107 (67)	23/107 (22)
BARD <2	98/191 (51)		93/191 (49)	BARD >2	81/107 (76)		26/107 (24)
FIB4 <1.3	133/191 (70)	47/191 (25)	8/188 (5)	FIB4 >2.67	22/107 (20)	53/107 (50)	32/107 (30)
NFS <–1.433	120/191 (64)	63/191 (33)	5/191 (3)	NFS >0.676	29/107 (27)	51/107 (48)	27/107 (25)
ADAPT <6.3	144/191 (75)		47/191 (25)	ADAPT >6.3	83/107 (78)		24/107 (22)
FIBC3 <–0.4	144/191 (75)		47/191 (25)	FIBC3 >–0.4	80/107 (75)		27/107 (25)
ABC3D <3	144/191 (75)		47/191 (25)	ABC3D >3	73/107 (68)		34/107 (32)

Performance of FIBC3 and ABC3D in real-world settings
We assessed the performance of FIBC3 and ABC3D in a range of pre-test probability scenarios that may be encountered across primary care and specialist care environments, where the prevalence of advanced fibrosis varies, to see if they were equivalent. The PPV and NPV were calculated across an advanced and mild fibrosis prevalence range between 5–50% (Table 6). We also stratified our validation cohort in different, clinically distinct, subpopulations and observed that performance was maintained across all sub-populations, with a reliable NPV for advanced fibrosis >74% (Table S4,5).

Performance of PRO-C3, FIBC3 and ABC3D as pre-screening tools prior to liver biopsy to support clinical trial recruitment
As there is also a need for tools to assist in pre-screening patients for clinical trials in NASH, we modelled the performance of PRO-C3 as pre-screening tools for entry into clinical trials of fibrosing steatohepatitis. Two target populations were modelled: (i) "tdNASH", defined as NAS ≥4 with at least 1 point each for steatosis, hepatocyte ballooning and hepatic inflammation and fibrosis stage ≥F2; and (ii) "tdNASH-Cirrhosis", defined as above but with fibrosis stage F4. For tdNASH, a PRO-C3 level >14.5 ng/ml had an AUROC of 0.68 (sensitivity 59%, specificity 69%, accuracy 64%). This was replicated in the validation cohort (n = 298), AUROC = 0.76, sensitivity 70%, specificity 68%, accuracy 69%. Similarly, a PRO-C3 level >16.5 ng/ml identified tdNASH-Cirrhosis with an AUROC of 0.68 (sensitivity 74%, specificity 67%, accuracy 68%). This was replicated in the validation cohort (n = 298), AUROC = 0.76, sensitivity 76%, specificity 61%, accuracy 63% (Table S2). The results for the FIBC3 and ABC3D scores in the complete cohort (n = 449) are shown in Table S6. In general, tests incorporating PRO-C3 performed well. The most accurate test for the detection of tdNASH was FIBC3 >-0.4 (71%). Phase II/III clinical trials that are currently recruiting will be informative for the further validation of these findings.

ABC3D to improve the accuracy of NFS and FIB4 scores
Although FIB4 and NFS are useful, the use of 2 cut-off thresholds leads to indeterminate results that fail to classify a substantial proportion of patients. For each diagnostic test we employed a method of sequential testing by applying the low and high cut-off values. The residual cohort of patients with NAFLD and indeterminate scores were then assessed with the ABC3D diagnostic algorithm to detect cases of advanced fibrosis (Tables S7,8). With the application of sequential testing, the accuracy improved from 52% to 70% in the cases involving indeterminate FIB4 scores and from 54% to 77% in the case involving indeterminate NFS scores.

Table 6. Predictive values of cut-offs at different prevalences of advanced and mild fibrosis.

Combined Cohort (n = 449)										
	Predictive values of cut-offs for different prevalences of advanced fibrosis (F>3); "Rule in" advanced fibrosis									
	FIBC3 >−0.4		ABC3D >3		FIB4 >2.67		NFS >0.676		ADAPT >6.3	
Prevalence of significant fibrosis (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
5	15	99	15	98	19	96	22	96	14	98
10	27	97	26	96	33	92	38	92	26	96
15	37	95	36	94	44	87	49	88	36	94
20	46	93	45	91	52	83	57	84	45	92
25	53	91	52	89	59	79	64	80	52	90
30	59	89	58	86	65	74	70	75	58	87
35	65	87	63	83	70	69	74	71	63	85
40	69	84	68	80	75	65	78	66	68	82
45	74	81	73	77	78	60	82	61	72	78
50	77	78	76	73	81	55	84	57	76	75
	Predictive values of cut-offs for different prevalences of mild fibrosis (F<2); "Rule in" mild fibrosis									
	FIBC3 <−0.4		ABC3D <3		FIB4 <1.3		NFS <−1.433		ADAPT <6.3	
Prevalence of mild fibrosis (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
5	25	99	12	98	11	98	14	98	12	97
10	42	97	23	97	21	95	26	95	22	94
15	53	96	32	95	30	93	36	92	31	91
20	62	94	40	93	38	90	45	90	39	88
25	68	92	47	91	46	87	51	87	46	85
30	73	90	53	88	51	84	57	84	52	81
35	78	88	59	86	57	81	63	80	58	78
40	81	85	64	83	62	78	68	76	63	74
45	84	82	69	80	67	74	72	73	68	70
50	87	79	73	76	71	69	76	68	72	65

PPV, positive predictive value; NPV, negative predictive value.

Discussion

NAFLD has an estimated global prevalence of 25%, which is predicted to rise internationally.[41–43] Associated mortality is directly proportional to fibrosis stage, with patients at ≥F3 being at highest risk.[2] Current non-invasive tests are suboptimal; therefore, there is a clear need for better diagnostic biomarkers to detect advanced fibrosis. Such tests could potentially aid diagnosis and risk stratification, as well as facilitate clinical trial pre-screening to reduce screening failure rates; all of which fall within the BEST *diagnostic* context of use.[11]

At present, the reference standard to assess severity of NAFLD is histological, using the semi-quantitative NASH CRN system.[37] However, it is generally accepted that inter- and intra-observer variability, and sampling error due to variability in the extent of fibrosis within the liver, may impair the accuracy and reproducibility of these histological assessments.[37,44,45] This implies a paradox that makes addressing the need for biomarkers all the more challenging: the histological reference standard, against which a biomarker is assessed, is inherently imperfect and unable to produce a completely error-free classification with respect to the presence or absence, or severity, of the target condition. Semi-quantitative histological grading conflates anatomical distribution of fibrosis with extent and imposes discrete categorical staging bins on what are continuous variables like collagen deposition.[37] This inevitably leads to discrepancies due to inter- and intra-observer judgement, especially at the margins. It also blunts sensitivity as semi-quantitative grades fail to recognise modest differences in severity that do not transition across predefined but arbitrary categorical boundaries. This phenomenon is well illustrated by the breadth of disease that is encompassed by stage F3 fibrosis in the NASH CRN classification[37] where histological portal-portal, central-central and/or portal-central bridging is the defining feature, yet no weight is given to density of collagen deposition or the number of "bridging" septae. The situation where an imperfect reference standard is used in place of a perfect standard, introduces "imperfect gold standard bias". This means that the performance of the new test may be under- or over-estimated and, even if it is in reality a better measure of disease, it never has the potential to generate an AUROC >0.90.[46] Although not unique to liver histopathology, such situations are methodologically challenging to address.[47]

Cognisant of these challenges, we report measurement of PRO-C3 levels in a large international cohort and incorporate this measure into novel diagnostic models that outperform numerous previously described blood-based tests that detect advanced fibrosis.[12–18]

Utility of PRO-C3 as a single diagnostic biomarker

Although isolated parameters seldom exhibit an adequate level of diagnostic accuracy and are unlikely to be a surrogate for the complex diagnostic information provided by liver biopsy, we assessed how PRO-C3 performed in this context of use. PRO-C3 performed moderately as a biomarker of advanced fibrosis, comparable to simple panels such as FIB4. Similarly, when used to screen patients for clinical trial recruitment, PRO-C3 accurately identified 65% of cases that were histologically eligible for current phase III trial recruitment (NASH with significant fibrosis). This moderate performance as a diagnostic biomarker may partially be explained by the biological process that generate PRO-C3 during collagen deposition, implying that PRO-C3 is most sensitive to active fibrogenesis rather than static collagen accumulation. Supporting this view, preliminary evidence suggests that PRO-C3 may aid the evaluation of patients with active collagen turnover.[48] In the present study we were unable to assess the value of PRO-C3 as a prognostic test, that could be used to enrich studies for cases at greatest risk of subsequent disease progression, or to monitor change in disease severity.

FIBC3 and ABC3D performance for risk stratification of fibrosing steatohepatitis

In light of the moderate performance of PRO-C3 as a single diagnostic biomarker, we assessed its value as part of a non-invasive fibrosis panel composed of routinely measured clinical and laboratory variables enhanced by inclusion of a single biomarker of fibrogenesis, PRO-C3. We report development and validation of FIBC3. Whilst not the first panel to incorporate these components, many of which are used within ADAPT,[29] the current study benefits from detailed development and validation in a large, international patient cohort where careful harmonisation of histological practice, coupled with central reviewing of biopsies, has been undertaken to minimise the potential impact of an imperfect reference standard. Overall, a FIBC3 threshold of >-0.4 correctly identified fibrosis status in 77% of patients in the total cohort. However, the diagnostic accuracy of ABC3D, a simplified panel, better adapted for use in clinical practice (at the bedside) rivalled this model with an accuracy of 75% and performed equivalently when assessed across different clinical sub-populations and consistently outperformed all other routinely used scores to which it has been compared. Thus, in contrast to FIB4, NFS or the PRO-C3 based ADAPT score, which require more complex formulas, this simple model can be easily calculated by summing 5 easy to assess clinical items, removing the need to access to a web-based calculator or App to aid patient risk stratification. Furthermore, in contrast to FIB4 or NFS, FIBC3 and ABC3D both have a single, optimised, risk-threshold value, without "indeterminate" results which would require further testing or liver biopsy to clarify disease severity.[19]

In the validation cohort, FIBC3 performed best, correctly identifying 75% of patients, with ABC3D more or less equivalent correctly identifying 72% of patients. In the full cohort of 449 patients, the FIBC3 model identified 254 patients as not having advanced fibrosis (at a threshold of less than -0.4) of which 217 were correctly classified. Therefore, in this "low-risk cohort" the FIBC3 model could have correctly avoided a liver biopsy in 85% of patients. Applying the same analysis to ABC3D, 267 patients were identified as 'low-risk' (score ≤3). In this cohort, 219 patients were correctly staged, thus potentially correctly avoiding biopsies in 82% of cases. Complex fibrosis panels also exist. They include markers of matrix turnover, such as the Enhanced Liver Fibrosis (ELF™) panel.[18] However, a recent meta-analysis has reported that ELF and NFS have very similar AUCs.[49] Extrapolating this observation to our data would imply that FIBC3/ABC3D (like the NFS) had comparable, if not better, diagnostic value than the more complex Fibrotest and ELF.

Potential to use ABC3D in primary care

The point performance of diagnostic tests in terms of PPV/NPV are affected by pre-test probability, which reflects the prevalence of disease in a specific clinical setting. The prevalence of advanced fibrosis in the current study cohort was 37% which is much higher than would be expected in a primary care setting. Indeed, population data, albeit limited, have found that

5.6% of the Dutch population have clinically significant fibrosis based on a VCTE liver stiffness >8 kPa.[50] Similarly, based on VCTE thresholds ≥6.8, ≥8.0, and ≥9.0 kPa prevalence estimates in the Spanish population were 9.0%, 5.8%, and 3.6%, respectively.[51] These levels contrast sharply to a tertiary referral centre where the prevalence of advanced liver disease is often well in excess of 10%, and frequently nearer 30%.[52–54] To model performance across a range of settings, we calculated PPV and NPV for prevalence levels of advanced fibrosis from 5–50%. The NPV for both FIBC3 and ABC3D were similar across a prevalence range of 5–15% and in excess of 90%. To explore performance of the models in specific patient subgroups, we split the cohort by gender, diabetes status, BMI, and patients with elevated or normal alanine aminotransferase levels. FIBC3 and ABC3D maintained high NPV in all subgroups, although sensitivity was lower in patients with a BMI <25 and non-diabetics.

Strengths and limitations

FIBC3 and ABC3D were developed using an international cohort of well-characterised, untreated patients with NAFLD, covering a wide spectrum of disease severity. Liver biopsies were read by expert histopathologists that belong to the EPoS consortium pathology group, a group that undertook extensive harmonisation procedures for NAFLD pathological assessment and demonstrated high kappa-value reproducibility.[45] Moreover, half of the biopsies across all sites were assessed centrally. While this certainly reduces the reader-related variability, it is still dependent on limitations intrinsic to histological classifications such as the semi-quantitative nature of fibrosis scoring and on sampling variability of the procedure. These limitations are common to all biomarkers that use biopsy as the reference standard. Our diagnostic model consists of readily available clinical and laboratory variables that are routinely determined in patients with NAFLD in outpatient appointments. PRO-C3 levels were also measured in a central College of American Pathologists certified lab by staff blinded to the clinical data, before results were sent to a separate, independent centre for statistical analysis. Protein finger print technology has been developed to produce a reliable assay for PRO-C3 measurement.[25] Our model, in comparison to previous complex biomarker panels (*e.g.* ELF or Fibrotest) includes only one variable that is not routinely measured in a clinical setting. To minimise the effects of inter-observer variability in fibrosis staging, half the cohort across all centres had centrally reviewed liver biopsies confirming high inter-observer agreement.

Although we have taken measures to minimise inter-observer variability in the histological scoring, and concordance between liver pathologists was very good, an element of variability cannot be fully excluded. We also acknowledge that percutaneous liver biopsy is prone to sampling error leading to mis-staging of disease severity. However, the key limitation, which is common to all biomarker studies that rely on histology, relates to the nature of the semi-quantitative scoring systems and how this conflates histological localisation of fibrosis and extent of collagen deposition. We also acknowledge that AUROCs are not perfect as a means for assessing diagnostic accuracy. ROC curves attribute equal weight to false positives and false negatives and do not provide information on predictive values, which may be of greater value in a clinical setting.[55] Our results require further independent validation in other patient populations, to critically assess these models' ability to discriminate fibrosis stage.

In conclusion, both FIBC3 and ABC3D are simple indices including accessible routine laboratory tests and a single marker of collagen turnover. We have shown that both can accurately differentiate mild to moderate fibrosis from bridging fibrosis and cirrhosis in patients with NAFLD. Given that the ABC3D model is much simpler to compute and can be done at the bedside, the ABC3D diagnostic index has the potential to be widely used for the identification of patients with significant/active fibrosing steatohepatitis who should undergo specialised liver explorations, closer monitoring and possibly, specific therapies. FIBC3 and ABC3D may also be used as pre-screening tools for therapeutic trials, potentially helping to minimise histological severity-related screening failure rates. However, this will require further prospective validation.

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Conflict of interest

MK, DL and MJN are employed at Nordic Bioscience, a privately-owned company responsible for the development of PRO-C3. MK and DL are stock holders in the company. SK is employed by and holds stock in GSK. The other authors have no relevant potential conflicts of interests and none of the authors have received any payment for the work described in this study.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study Concept and Design QMA, SK; Data collection QMA, MB, DT, JMS, VR, EB, SP, CPO, OG, MY, SM, PB, MJN, MK, DL; Statistical analysis MB; Drafting initial manuscript MB, QMA; All authors reviewed the manuscript, revised the manuscript for important intellectual content and approved the final manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhepr.2019.06.004.

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Appendix C: Plasma cell-free DNA methylation- a liquid biomarker of hepatic fibrosis

Plasma cell-free DNA methylation: a liquid biomarker of hepatic fibrosis

We recently reported dynamic epigenetic markers of fibrosis detectable in patients' plasma that may have utility in non-invasive diagnosis and staging of fibrosis in patients with chronic liver disease.[1] Specifically, we uncovered DNA methylation markers at the human PPARγ promoter detectable in circulating cell-free DNA (ccfDNA) that display differential methylation densities. Remarkably, PPARγ hypermethylation correlated with progression to cirrhosis in alcoholic liver disease (ALD) and with specific stages of liver fibrosis in non-alcoholic fatty liver disease (NAFLD). Furthermore, ccfDNA signatures were traced back to the molecular pathology in fibrotic liver tissue, providing a biomarker of the underlying pathological process and defining hepatocytes as the source of hypermethylated DNA found in plasma.[1]

The original study posed several important outstanding questions: (1) Can ccfDNA methylation be used as a biomarker of fibrosis in liver diseases of other aetiologies? (2) Does the presence of hepatocellular carcinoma (HCC) alter the biomarker in plasma? (3) Does presence of fibrosis in other organs generate similar biomarker profiles?

In the present letter, we answer these questions and demonstrate the broader utility of DNA methylation at three CpG dinucleotides within PPARγ promoter in several new patient cohorts (figure 1A and table 1). Employing pyrosequencing we detect hypermethylation at all three CpGs in ccfDNA from a cohort of patients suffering from cirrhosis caused by chronic HBV infection (figure 1B–D). The level of hypermethylation resembled that found in patients with cirrhotic NAFLD and ALD in

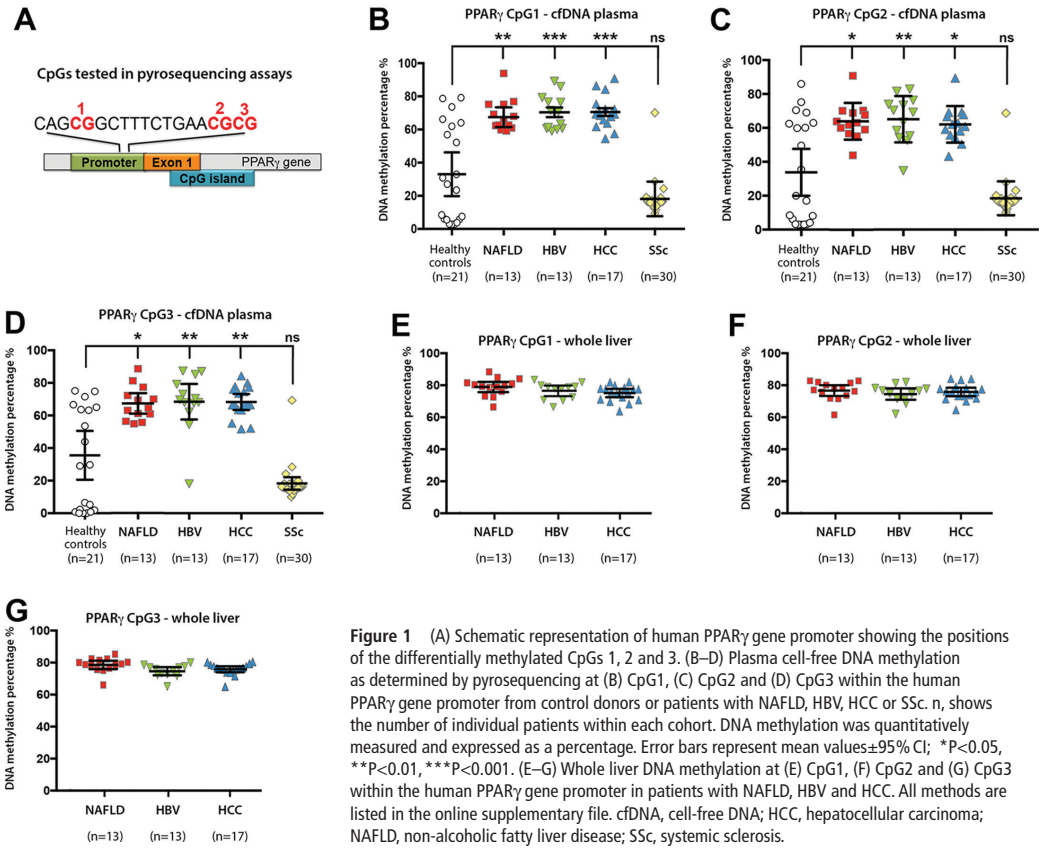

Figure 1 (A) Schematic representation of human PPARγ gene promoter showing the positions of the differentially methylated CpGs 1, 2 and 3. (B–D) Plasma cell-free DNA methylation as determined by pyrosequencing at (B) CpG1, (C) CpG2 and (D) CpG3 within the human PPARγ gene promoter from control donors or patients with NAFLD, HBV, HCC or SSc. n, shows the number of individual patients within each cohort. DNA methylation was quantitatively measured and expressed as a percentage. Error bars represent mean values±95% CI; *P<0.05, **P<0.01, ***P<0.001. (E–G) Whole liver DNA methylation at (E) CpG1, (F) CpG2 and (G) CpG3 within the human PPARγ gene promoter in patients with NAFLD, HBV and HCC. All methods are listed in the online supplementary file. cfDNA, cell-free DNA; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; SSc, systemic sclerosis.

our original study. However, since the HBV cohort was of another ethnicity to our original UK-based patients with NAFLD and ALD, we also measured methylation density in a Turkish NAFLD cohort, which was mirroring those detected in the HBV cohort. Our new data also demonstrate that presence of HCC with chronic liver disease does not alter the specificity of the DNA methylation markers for detection of liver fibrosis (figure 1B–D). As we had access to explant liver tissue from patients with NAFLD, HBV and HCC, we determined methylation densities in the liver (figure 1E–G). A high similarity was observed between the degree of DNA methylation at PPARγ gene promoter in ccfDNA and in the patient-matched liver tissues. We found a significant spread of values for DNA methylation in the healthy control ccfDNA, this being in contrast with our original UK-based study in which low-level methylation density was consistent across individuals within the control

Table 1 Characteristics of patient cohorts used in the study

	Age (years)	Gender (male/female)	BMI (kg/m^2)	Diabetes (%)	ALT (IU/L)	AST (IU/L)
NAFLD cohort	56 ± 7	10/3	29.8±3.2	69	33±23	54±36
Hepatitis B cohort	51±7	10/3	26.5±2.4	38	47±50	80±64
HCC cohort	57±7	16/1	27.5±4.2	29	55±36	65±53

Systemic sclerosis cohort (n=30)	Age (years)	Gender (male/female)	BMI (kg/m^2)	Diffuse cutaneous limited SSc	Disease duration (years)	Heart involvement	Lung involvement	DLCO (%)	Antinuclear antibody-positive	Anticentromere antibody-positive	Antitopoisomerase I antibody-positive
	55±14	10/20	26±3.8	12 (40%)	7.5±4	2 (7%)	11 (37%)	71.7±17	30 (100%)	11 (37%)	12 (40%)

Notes: Viral hepatitis in HCC cohort: HBV-positive, n=8; HCV-negative, n=2; HBV-positive and HCV-positive, n=3.
Data expressed as mean±SD or median (range).
BMI, body mass index; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; SSc, systemic sclerosis.

group. We are unable to explain this wider spread of methylation densities in the Turkish cohort, but cannot rule out an undetected liver disease in the apparently 'healthy' controls that display elevated ccfDNA methylation.

We next determined if hypermethylation is specific to fibrosis of liver origin. To this end, we quantified ccfDNA methylation in a cohort of patients with limited and diffuse systemic sclerosis (SSc) who have various combinations of skin, lung and kidney fibrosis, but no hepatic fibrosis.[2] All three CpG sites in SSc were relatively hypomethylated (figure 1B–D), with similar methylation densities between individual patients with SSc. All methods relating to the study are listed in 'online supplementary materials and methods 1'.

This important validation study supports our original hypothesis that hypermethylation at the PPARγ gene promoter is a marker for fibrotic progression of chronic liver disease and holds true for viral, alcoholic and metabolic disease aetiologies. As fibrosis in other organs does not generate a similar epigenetic signature, it is likely that the PPARγ hypermethylation specifically reflects a liver pathology. The ability to detect and quantify hypermethylation at the promoter of the PPARγ in ccfDNA as a new liquid biomarker that specifically reports the fibrotic progression of liver diseases of multiple aetiologies offers the potential for a cost-effective blood-based liquid biomarker of liver fibrosis.

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