UNIVERSITY OF SOUTHAMPTON

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Biomarkers in the assessment and management of patients with chronic liver disease

by

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ABSTRACT

FACULTY OF MEDICINE
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BIOMARKERS IN THE ASSESSMENT AND MANAGEMENT OF PATIENTS WITH CHRONIC LIVER DISEASE

By Kevin John Fagan

Due to the rising prevalence of chronic liver disease (CLD) and its increasing burden on the health care system, the development and validation of biomarkers to aid in the assessment and management of patients is paramount. The aim of this thesis was to establish the need for biomarkers in patients with CLD and assess known and novel biomarkers that have been suggested for the assessment of alcohol intake, liver fibrosis and management of patients with decompensated cirrhosis.

Alcohol, a significant primary and comorbid cause of liver injury, can impede therapeutic strategies or expedite disease progression. My work confirmed the need for an objective alcohol biomarker, corroborating previous findings that failure to recognise or acknowledge significant alcohol consumption remains common in clinical practice. Assessment of the most specific serum biomarker of sustained alcohol intake, carbohydrate deficient transferrin, demonstrated its poor sensitivity for detecting heavy alcohol consumption in patients with CLD, influenced by body mass index, gender and stage of liver fibrosis.

Non-invasive detection and quantification of hepatic fibrosis is important in the identification, assessment and management of patients with CLD. The performance of the ELF test was assessed in a cohort of patients with CLD and demonstrated to be good at detecting advanced fibrosis using the manufacturer's cut-off (≥9.8). The performance of ELF test was negatively influenced by inflammation and age, but performed well in the presence of steatosis. This latter finding has not previously been described and is a significant finding in view of the global NAFLD epidemic.

Patients with cirrhosis and ascites have a significant increase in morbidity and mortality and my work supports the need for better identification of patients with poor outcomes and coordination of patient care. Assessment of bacterial DNA, extracted from ascites, could replace culture based techniques and predict patients with poor outcomes. There was evidence of impaired innate immune function and the ascites bacterial communities reflected the intestinal dysbiosis that occurs in patients with cirrhosis. Bacterial DNA may therefore further increase our knowledge of the pathogenesis of infection in cirrhosis and facilitate development of therapies and identification of other biomarkers for use in clinical practice.

Overall the work in this thesis has proven the need for development and validation of biomarkers for the management of patients with CLD and highlighted future studies that may eventually lead to better clinical outcomes for patients with CLD.

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Academic Thesis: Declaration of Authorship

I, **Kevin John Fagan** declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

"Biomarkers in the assessment and management of patients with chronic liver disease"

I confirm that:

1. This work was done wholly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Either none of this work has been published before submission, or parts of this work have been published as below:

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Chapter 6. Fagan KJ, Pretorius C, Horsfall L, Irvine K, Wilgen U, Choi K et al. ELF score ≥9.8 indicates advanced hepatic fibrosis and is influenced by age, steatosis and histological activity. *Liver International. 2015;**35**:1673-1681*

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Statement of contribution to jointly published work

For the published manuscripts relating to this thesis I have contributed to the conception and design of the projects, acquired relevant data and samples, helped perform the laboratory analysis, completed data analysis and interpretation, wrote the first drafts of the manuscripts and approved final versions for publication.

Statement of contribution by others

Professor Elizabeth Powell and Dr Katharine Irvine provided support with conception and design of projects, laboratory analysis, interpretation of data and critical review of the manuscripts. Dr Emma Ballard and Professor Peter O'Rourke provided statistical advice and support. Contributions by others to the published work are listed in each of the results chapters.

Signed: ……... Signed: ……..

Date: …… Date: ……

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Presentations by the candidate relevant to the thesis

Oral Presentations

1. Gastroenterological Society of Queensland meeting, Coolum, June 2012.
   "Carbohydrate Deficient Transferrin as a biomarker of alcohol excess in patients with chronic liver disease: BMI affects diagnostic sensitivity."
   Young investigator award finalist.

2. Diamantina Health Partners Inflammation, Infection and Immunity in Digestive Disease Research Workshop, May 2014.
   "The ascites microbiome and its association with clinical outcomes in patients with decompensated cirrhosis."

3. Gastroenterological Society of Queensland meeting, Noosa, May 2014.
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Poster Presentations

1. Australian Gastroenterology Week, Adelaide, October 2012.
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2. European Association for the Study of the Liver, Amsterdam, April 2013.
   "BMI but not stage or aetiology of non-alcoholic liver disease affects the diagnostic utility of carbohydrate deficient transferrin."
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3. Princess Alexandra Health Symposium, Brisbane, August 2013.
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4. Australian Gastroenterology Week, Melbourne, October 2013.
   "Factors associated with mortality and unplanned hospital readmissions in patients with ascites."

5. The Asian Pacific Association for the Study of the Liver, Brisbane, March 2014.
   "Diagnostic Sensitivity of Carbohydrate Deficient Transferrin in Heavy Drinkers."

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   "An ELF score of ≥9.8 can identify advanced fibrosis in Australian patients with chronic liver disease."

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Abbreviations

5-HTOL	5-Hydroxytryptophol
ADH	Alcohol dehydrogenase
ALD	Alcohol related liver disease
ALT	Alanine aminotransferase
AMPs	Antimicrobial peptides
APRI	Aspartate aminotransferase to platelet ratio index
ARFI	Acoustic radiation force impulse
AST	Aspartate aminotransferase
AUDIT	Alcohol Use Disorders Identification Test
AUROC	Area under the receiver operating characteristic curve
BC	Bray-Curtis
bMAST	Brief Michigan Alcoholism Screening Test
BMI	Body mass index
BT	Bacterial translocation
CDT	Carbohydrate deficient transferrin
CFU	Colony forming units
CirCom	Cirrhosis-specific comorbidity scoring system
CKD	Chronic kidney disease
CLD	Chronic liver disease
CPA	Collagen proportional area
CRP	C-reactive protein
CTP	Child-Turcotte-Pugh
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
eGFR	Estimated glomerular filtration rate
ELF	Enhanced liver fibrosis
EtG	Ethylglucuronide
EtS	Ethyl sulfate
FAEE	Fatty acid ethyl esters
FAST	Fast Alcohol Screening Test
FBS	Fetal bovine serum
FN	False negative
FP	False positive
GP	General practitioner
HA	Hyaluronan
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
IFN-γ	Interferon-gamma

IgA	Immunoglobulin A
INR	International normalised ratio of the prothrombin time
LBW	Lean body weight
MAP	Mean arterial blood pressure
MAST	Michigan Alcoholism Screening Test
MCV	Mean corpuscular volume
MELD	Model for end-stage liver disease
MMP	Matrix metalloproteinase
MRE	Magnetic resonance elastography
MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
NHMRC	National Health and Medical Research Council
NK	Natural killer
NMS	Non-metric Multidimensional scaling
NO	Nitric oxide
NPV	Negative predictive value
OELF	Original European liver fibrosis
PAST	Palaeontological Statistics
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PCT	Procalcitonin
PEth	Phosphatidylethanolamine
PIIINP	N-terminal peptide of procollagen type III
PPV	Positive predictive value
ROC	Receiver operating characteristic
rRNA	Ribosomal ribonucleic acid
RWA	Recommended maximum weekly allowance
SAAG	Serum-ascites albumin gradient
SBP	Spontaneous bacterial peritonitis
SIBO	Small intestinal bacterial overgrowth
SIRS	Systemic inflammatory response syndrome
SSF	Subsinusoidal fibrosis
TE	Transient elastography
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TN	True negative
TNF	Tumour necrosis factor
TP	True positive
Vd	Volume of distribution
WHO	World Health Organization
γ-GT	Gamma glutamyl-transferase

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Chronic liver disease (CLD) is a major global health burden that is increasing secondary to the increasing prevalence of non-alcoholic fatty liver disease (NAFLD), hazardous alcohol consumption, and aging of the viral hepatitis B (HBV) and C (HCV) infected cohorts. In Australia, liver disease including fatty liver affects more than a quarter of the population and healthcare costs for treating liver disease were estimated to be $432 million in 2012[1]. CLD has a substantial latency period, during which subjects are often asymptomatic despite progressive fibrosis. The majority of morbidity and mortality associated with liver disease occurs in subjects with advanced fibrosis, particularly cirrhosis, who are at risk of developing hepatocellular cancer or complications of end stage liver disease (e.g. ascites and spontaneous bacterial peritonitis (SBP)).

The progression of all four of the main aetiologies of liver disease is potentially preventable. It is therefore important to detect factors that can impede therapeutic strategies or expedite disease progression, such as alcohol, which is a significant primary and comorbid cause of liver injury. It is also essential that subjects who have progressed to advanced liver fibrosis are identified so their management can be optimised and surveillance procedures for hepatocellular cancer, varices, bone mineral density implemented. For subjects with decompensated cirrhosis it is essential to recognise those at higher risk of poor outcomes, to improve management and use of the health care system.

Currently routine blood tests are often used in the screening and stratification of patients with liver disease, but they are generally not sensitive or specific. The development and validation of biomarkers is thus paramount to improving the health outcomes of patients with CLD, since this will enable better more reliable detection, monitoring and management of factors identified to be integral to disease progression or poorer outcomes.

1.1.1 Ideal characteristics of biomarkers

A biomarker is defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention"[2]. They are often divided into direct, reflecting physiological processes, or indirect, reflecting functional alterations due to damage or dysfunction. Biomarkers should ideally be acceptable to the patient, safe, easy to measure, reproducible, cost effective and reliable. The perfect biomarker would be both sensitive and specific with a high negative and positive predictive value (Figure 1.1). However, the accepted sensitivity or specificity of the biomarker will vary depending on the clinical situation and the outcome that may occur if the test fails.

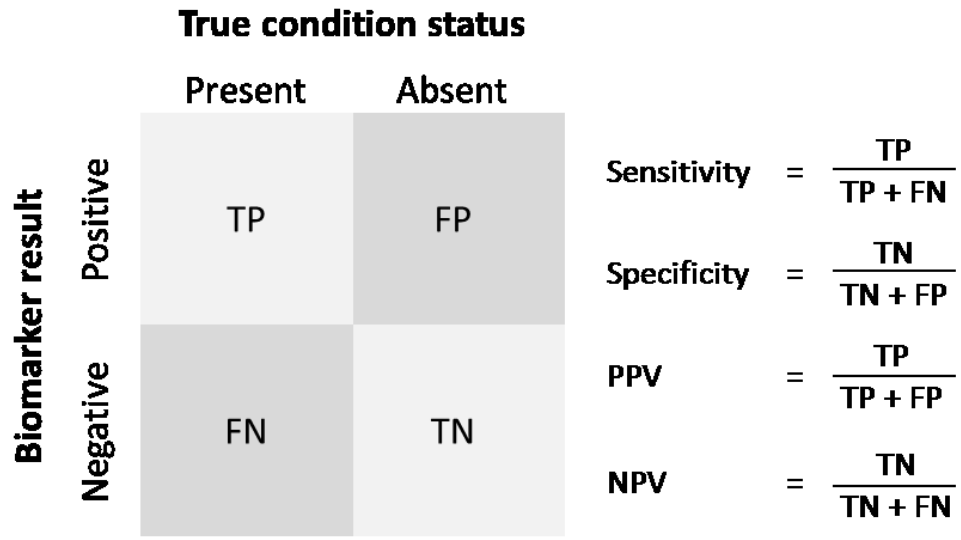


Figure 1.1. Performance characteristics of biomarkers. *Abbreviations: TP, true positive; FP, false positive; FN, false negative; TN, true negative; PPV, positive predictive value; NPV, negative predictive value.*

Receiver operating characteristic (ROC) curve estimation is used to determine cut-off values to identify the accepted sensitivity and specificity of the biomarker in comparison to the gold standard. The most useful biomarkers are typically those with the largest area under the ROC curve (AUROC), with the perfect test having an AUROC of 1. AUROC can be used to further categorise performance of biomarkers as excellent (0.90–1.00); good (0.80–0.90); fair (0.70–0.80); poor (0.60-0.70); failed (0.50-0.60).

1.2 Assessment of alcohol consumption

Alcohol has been consumed since the beginning of recorded history and its causal role for increased morbidity and mortality established[3]. It is related to more than 60 different medical health problems[3] and attributed to 6.2% of all male deaths and 1.1% of female deaths worldwide[4]. Although developed countries (North America, Western Europe, Japan, Australia) have a lower mortality than Eastern Europe, Central Asia and developing countries, their total disease burden related to alcohol is 6.8%, of which more than half is due to alcohol use disorders[5], which are defined as[6]:

- **Hazardous drinking.** A pattern of use that is of public health significance despite the absence of any current disorder in the individual user.
- **Harmful drinking.** A pattern of alcohol consumption causing health problems directly related to alcohol. The damage may be physical or mental. Harmful use commonly but not invariably has adverse social consequences.
- **Alcohol dependence:** A cluster of behavioural, cognitive and physiological phenomena that develop after repeated use that typically include impaired control of alcohol use, with drinking becoming habitual and problematic, persisting in its use despite harmful consequences. There is a higher priority given to alcohol than to other activities and obligations, increased tolerance and sometimes a physical withdrawal state.

The National Health and Medical Research Council (NHMRC) recommends healthy men and women drink no more than 2 standard drinks on any day (1 standard drink contains 10g alcohol), to reduce lifetime risk of harm from alcohol-related disease or injury and that they drink no more than 4 standard drinks on a single occasion to reduce the risk of alcohol-related injury arising from that occasion[7]. In Australia, *per capita* consumption of pure alcohol was estimated to be 10.1 litres in 2012, which is considered high by international standards[4,8].

Unsurprisingly, alcohol remains an important primary cause of liver injury in Australia. Epidemiological data suggests that liver injury is more likely to occur at 140g/week for women and 210g/week for men[9-12]. However, in subjects with other CLD such as NAFLD and viral hepatitis, where it can act synergistically[13,14], the safe limit of alcohol remains unclear[15].

Detection of hazardous or harmful alcohol consumption, in addition to alcohol dependence, is therefore essential to enable prevention and earlier management of alcohol related liver problems. There is also a need to confirm alcohol abstinence in patients being considered for liver transplantation. Unfortunately, in clinical practice, failure to recognise or acknowledge significant alcohol consumption remains common, with studies reporting that general practitioners or hospital doctors identify only 30% of subjects with hazardous or harmful patterns of alcohol use[16-21]. Development and use of screening surveys and biomarkers are therefore important in identification of subjects drinking alcohol at at-risk levels.

1.2.1 Alcohol screening surveys

Obtaining an accurate alcohol history is the most important means for detecting significant alcohol use and established approaches include the use of frequency or quantitative alcohol histories and/or structured screening instruments. These processes should be undertaken sensitively and respect the patient's privacy, dignity and confidentiality. They need to be sensitive and reasonably specific but brief and easy to use and/or score.

1.2.1.1 Alcohol frequency and quantity tools

Frequency is the simplest measure of alcohol consumption used, but it does not allow calculation of volume. Instruments like "The Time Line Follow Back" are the current gold standard for quantifying lifetime alcohol consumption, but these are not practical in routine clinical practice[22]. Other measures used include: graduated frequency; short term recall; and quantity-frequency:

- **Graduated frequency measure.** Groups the number of drinks consumed on an occasion into categories and then works progressively backwards from the maximum category to the lowest to determine how often the subject drank that amount over the last year. This method can be difficult to recall and time consuming for the clinical environment.
- **Short term recall measure.** Focus on how much alcohol the patient has drunk over a shorter period e.g. last week or month. It is easy to administer and for the patient to recall, but is a weak tool as it can miss patterns of drinking, particularly in occasional drinkers. Ideally alcohol consumption should be assessed over a 12 month period in order to identify problems related to alcohol[23].
- **Quantity-frequency measure.** Considered one of the most universal and practical tools for measuring alcohol consumption it comprises the daily average consumption (grams/standard drinks per day) of alcohol and the number of drinking days per week (or month). It is very simple, but can miss the true total average alcohol consumed. To improve its sensitivity further questions can target what specific beverages are consumed and whether subjects have a history of episodic or binge drinking. The Australian Government Department of Health and Ageing guidelines (2009) recommend the quantity-frequency measure to detect levels of alcohol consumption in excess of NHMRC guidelines in the general population[16].

In addition to quantity-frequency questions, social and psychological consequences (e.g. insomnia, anxiety, depression, evidence of trauma, drink driving offences) should also be assessed. Correspondingly, such complaints should prompt an alcohol history, as this is the commonest way patients with alcohol related problems present[24,25].

1.2.1.2 Structured instruments

Structured instruments have been demonstrated to perform better than quantity-frequency questions[26], possibly reflecting the incorporation of questions regarding social and psychological consequences. Structured questionnaires have been developed for certain conditions e.g. pregnancy ("TWEAK")[27] and clinical settings e.g. primary care ("AUDIT")[28], emergency department ("FAST")[29]. In the past structured instruments mainly targeted alcohol dependence and abuse, but due to improved treatment strategies the focus has now shifted to include hazardous or harmful drinking, to enable earlier identification and intervention.

1.2.1.2.1 Hazardous and harmful drinking

A number of screening questionnaires have been developed, of which the Alcohol Use Disorders Identification Test (AUDIT) is recommended by the NHMRC to identify current hazardous alcohol consumption in the general population[28]. Developed by the World Health Organization (WHO), it contains 10 questions that cover the domains of hazardous drinking (Q1-3), dependence symptoms (Q4-6), and harmful alcohol use (Q7-10). (Table 1.1)

AUDIT was designed specifically to detect recent hazardous and harmful drinking in a primary care setting[28]. Subsequently it has been shown to be useful in the general population[30] and to indicate active alcohol misuse and dependence disorders[31], deviations from its original purpose. It has been validated for use across different cultural groups and shown to have increased accuracy relative to other screening questionnaires for detecting hazardous and harmful alcohol consumption[32]. The initial study demonstrated that a score ≥8 had a sensitivity of 92% and specificity of 94% for hazardous or harmful alcohol use[28]. Subsequent independent studies demonstrated that AUDIT is a reliable and valid screening test, reporting a sensitivity between 57% and 97% and specificity between 78% and 96%[32-35].

Table 1.1. Alcohol Use Disorders Identification Test Questionnaire[28].

Question	0	1	2	3	4
1) How often do you have a drink containing alcohol?	Never	Monthly or less	2 to 4 times a month	2 to 3 times a week	≥4 times a week
2) How many “standard” drinks containing alcohol do you have in a typical day when you are drinking?	1 or 2	3 or 4	5 or 6	7 to 9	10 or more
3) How often do you have six or more drinks on one occasion?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
4) How often during the last year have you found that you were not able to stop drinking once you started?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
5) How often during the last year have you failed to do what was normally expected from you because of drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
6) How often during the last year have you needed a drink in the morning to get yourself going after a heavy drinking session?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
7) How often during the last year have you had a feeling of guilt or remorse after drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
8) How often during the last year have you been unable to remember what happened the night before because you had been drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
9) Have you or someone else been injured as a result of your drinking?	Never		Yes, but not in the last year		Yes, during the last year
10) Has a relative, a friend, a doctor or other health worker been concerned about your drinking or suggested you cut down?	No		Yes, but not in the last year		Yes, during the last year

Table 1.1 note. The overall score is calculated by adding the score for each answer, using the scores assigned at the top of each column.

Detecting subjects before they become alcohol dependent is important as it enables earlier implementation of prevention and management strategies, which are effective in reducing alcohol consumption[36]. Table 1.2 presents suggested interventions for each risk level based upon the AUDIT score[6].

Table 1.2. Suggested interventions for each risk level based upon AUDIT scores[6].

Risk level	Intervention	AUDIT score
Zone 1	Alcohol education	<8
Zone 2	Brief intervention Periodic re-assessment	8-15
Zone 3	Brief intervention Regular monitoring	16-19
Zone 4	Diagnostic assessment (specialist) Treatment	20-40

Table 1.2 note. Clinical judgement should be exercised in interpretation of AUDIT score.

A significant barrier to the application of screening tools is time constraints[37]. The AUDIT score has subsequently been modified (AUDIT-C) to employ only 3 questions to make it more practical (Table 1.3). Scored 0-12, it is a scaled marker of alcohol consumption and risk of alcohol use disorder[38-40]. In the initial study of 243 patients, AUDIT-C performed similarly to the full AUDIT, but was not as good at detecting active alcohol abuse or dependence[41]. However, more recent studies have demonstrated that inconsistencies can occur between the test score and the reported alcohol consumption[42]. Furthermore, AUDIT-C and other shorter instruments e.g. Fast Alcohol Screening Test (FAST)[29] are not as accurate at allocating subjects to one of the four main drinking categories: low risk, increasing risk (hazardous), higher risk (harmful), or possibly dependent. Despite their poorer performance compared to the full AUDIT, they are more accepted in busy clinical practice and thus more frequently employed.

Table 1.3. AUDIT-C questionnaire[41].

Question	0	1	2	3	4
How often did you have an alcoholic drink in the past year?	Never	Monthly or less	2 to 4 times a month	2 to 3 times a week	≥4 times a week
How many standard alcoholic drinks did you have on a typical day in the past year?	1 or 2	3 or 4	5 or 6	7 to 9	10 or more
How often did you have six or more drinks on one occasion in the past year?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily

Table 1.3 notes. The overall score is calculated by adding the score for each answer, using the scores assigned at the top of each column. Positive test for hazardous drinking or active alcohol use disorder: men >4; women >3.

1.2.1.2.2 Alcohol dependence and abuse

Before attention turned increasingly toward prevention and management, instruments were more directed to diagnosis of alcohol dependence and abuse. Rather than focussing on alcohol consumption over the last year, these generally enquire over the lifetime of the subject. The CAGE questionnaire[43] was one of the most commonly used screening tests and includes 4 simple questions:

- Have you ever felt you need to *C*ut down your drinking?
- Have people *A*nnoyed you by criticising your drinking?
- Have you ever felt *G*uilty about your drinking?
- Have you ever had an *E*ye opener (early morning drink to steady your nerves)?

The CAGE questions, based on symptoms of dependence, perform better at identifying patients with alcohol abuse and dependence than AUDIT[32] and laboratory tests (e.g. plasma alcohol level, mean corpuscular volume (MCV),

gamma-glutamyl transferase (γ-GT)[44]). Ascertaining a history of alcohol dependence or abuse is essential in patients with liver disease as risk of recidivism is significant. Furthermore for patients with alcohol dependence or abuse, determining the severity is helpful to inform treatment planning and clinical decision making[45]. AUDIT[28], Michigan Alcoholism Screening Test (MAST)[46] and Brief MAST (bMAST)[47] are commonly used. The latter is a shorter version (10 questions) (Table 1.4) of the MAST (25 questions) that is as effective at indexing severity of alcohol problems[19,47] and is more practical due to its brevity. It has also been proven to be efficient and effective at screening, including in the acute assessment of patients[45].

Table 1.4. The Brief Michigan Alcoholism Screening Test[47].

Questions	No	Yes
Do you feel you are a normal drinker?	2	0
Do friends and relatives think you are a normal drinker?	2	0
Have you ever attended a meeting of AA?	0	5
Have you ever lost friends or girlfriends/boyfriends because of drinking?	0	2
Have you ever been in trouble at work because of drinking?	0	2
Have you ever neglected your obligations, your family, or your work for 2 or more days in a row because of drinking?	0	2
Have you ever had “DTs”, severe shakes, heard voices or hallucinated after heavy drinking?	0	2
Have you ever gone to anyone for help about your drinking?	0	5
Have you ever been in hospital because of drinking?	0	5
Have you ever been arrested for drink driving?	0	2

Table 1.4 notes. Score >6 indicate high probability of alcohol dependence.

1.2.1.3 Limitations of screening surveys

Despite screening questionnaire methods being more valid and cost effective than blood screening methods[33], uptake of alcohol screening questionnaires is low[48,49]. Evidence suggests that practitioners refrain from challenging patients regarding their alcohol use to maintain doctor-patient relationships[50], potentially

because they feel it could be perceived as an attack on the patient's integrity[51]. However, it is reported that patients expect to be asked about alcohol consumption and associate it with a higher quality of care[52]. Another barrier to the use of screening tools is the ambivalence of doctors, as many do not feel that histories will be reliable[53,54]. This may reflect the practitioners prior use and confidence with these tools[18], but in their support, a prior study did demonstrate that electronic administration of the AUDIT-C was more likely to identify at-risk drinking than the same screening questionnaire administered in person or on paper[55].

1.2.2 Biomarkers of alcohol use

Subjects may struggle with recall or be defensive and understate alcohol intake, particularly if it may be viewed as excessive or problematic and/or they are not seeking treatment for it. The use of measurable clinical biomarkers provides a more objective way to evaluate drinking behaviour. These are intended to complement, not replace, structured surveys. Potentially they facilitate obtaining a more realistic history and can be used for screening, detection of relapse and to monitor treatment response. Alcohol biomarkers can be divided into direct (alcohol or its metabolites after normal biological processes) or indirect (markers of alcohol-induced tissue damage or dysfunction).

1.2.2.1 Direct biomarkers

Most alcohol absorbed undergoes first pass metabolism in the liver or stomach via oxidative pathways using alcohol dehydrogenase (ADH)[56-58]. Other tissues e.g. the brain also contribute to oxidative metabolism by the enzymes cytochrome P450 and catalase (Figure 1.2). Non-oxidative metabolism of alcohol is minimal, but results in the formation of products that can also be used as biomarkers. A small percentage (2-5%) of alcohol absorbed is not metabolised and excreted unchanged in the urine, sweat or breath.

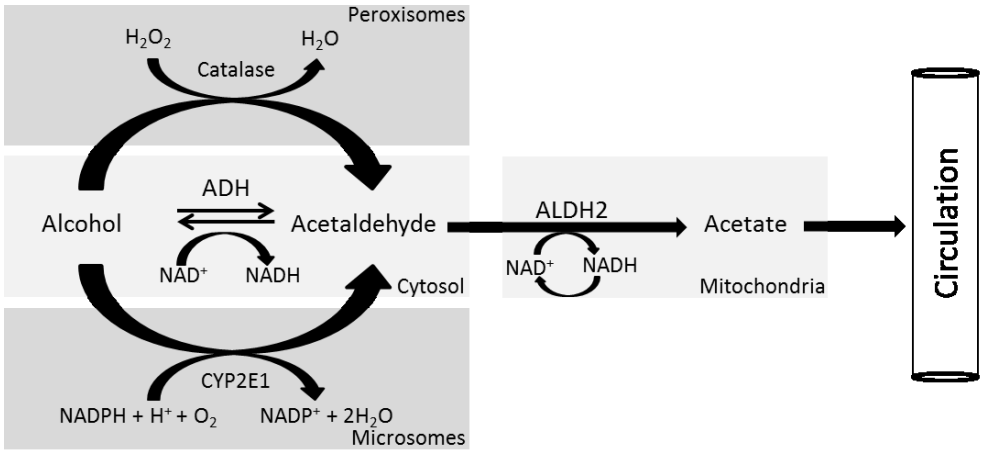


Figure 1.2. Oxidative pathways of alcohol metabolism. *Abbreviations: ADH, alcohol dehydrogenase; ALDH2, aldehyde dehydrogenase 2; H_2O_2, hydrogen peroxide; H_2O, water; NAD+/H oxidized/reduced nicotinamide adenine dinucleotide; CYP2E1, cytochrome P450 2E1* (adapted from [59]).

1.2.2.1.1 Alcohol concentration

Alcohol is the primary biomarker of alcohol use and can be measured in the blood, saliva, breath or urine. The peak time to detection after one drink ranges from approximately <30 minutes to 3 hours depending on the biological media: breath, <0.5-1 hour; blood, 1-2 hours; saliva, 1-3 hours; urine, 2-3 hours. Due to its rapid elimination, assessment 6-8 hours after ingestion is difficult, thus it is only useful for detecting acute alcohol consumption. However, when combined with clinical signs it can be used to infer long-term drinking habits[60]. The alcohol level is affected by how quickly it is metabolised, which can be increased e.g. in alcohol dependent subjects[61] or reduced e.g. in Asian people[62].

1.2.2.1.2 Metabolites of alcohol

A number of alcohol metabolites have been identified that can be used as biomarkers. Many of these are not currently suitable for clinical practice due to methodology and cost. Some of the metabolites most commonly used in research are discussed below.

- **Ethylglucuronide (EtG).** A small amount of alcohol (<0.1%) is removed by a conjugation reaction, resulting in the formation of EtG[63]. EtG is eliminated slower than alcohol and the concentration peak is measurable at different times in the blood (3-8 hours), urine (20-100 hours) and hair (several months)[64-66]. It is highly sensitive and specific to alcohol consumption and is regarded as a very reliable indicator of recent drinking[67]. However, incidental alcohol exposure e.g. cooking, mouthwash, over-the-counter cold medications can influence EtG[68,69], although this is reportedly uncommon and appears to rely largely on the cut-off applied[70]. Urine measurements are the most commonly utilised, which can also be influenced by yeast contamination in the presence of glucose (diabetes), bacterial infection, and dilution[65,71,72]. Currently the test is not suitable for clinical practice, mainly due to cost.
- **Ethyl sulfate (EtS).** Another minor metabolite of alcohol (<0.1%) is formed from the sulfate conjugation of alcohol, catalysed by cytosolic sulfotransferase[73]. EtS has a longer window of detection than EtG, to which it is highly correlated[73]. Both can be measured simultaneously which may allow verification of results[74], although this may be redundant with the advent of enzyme-linked immunosorbent assay tests for EtG[75]. Difficult methodology limits clinical interest.
- **5-Hydroxytryptophol (5-HTOL).** This is a minor metabolite of serotonin under normal physiologic conditions that has a dose-dependent increase with alcohol consumption, becoming detectable at approximately >50g alcohol[76]. Expressed as a ratio to 5-hydroxyindole-3-acetic acid, it has high sensitivity and specificity and is reportedly not influenced by age, gender, or liver disease[76]. It has a short detection window (6-24 hours) after cessation of alcohol and thus is only useful in detecting recent alcohol consumption[77].
- **Phosphatidylethanolamine (PEth).** This is an abnormal phospholipid that is generated from a phospholipase-D-catalysed reaction in the presence of alcohol[78]. It is detectable in the blood after consumption of approximately 1000g of alcohol, over a 2 week period[79,80]. Basal levels are achieved after about 15 days of abstinence[67]. PEth is highly specific

(100%) and sensitive (94.5-100%)[81-83]. It is a reliable test for detecting moderate to heavy alcohol use and its validity was recently reported to remain high regardless of age, gender or liver disease severity[84]. However, due to challenging detection methodology it is currently not suitable for clinical practice.

- **Fatty acid ethyl esters (FAEE).** Non-oxidative metabolites of ethanol are produced by esterification of ethanol with fatty acids e.g. ethyl palmitate, ethyl oleate and ethyl stearate[85]. FAEE are present in serum shortly after alcohol consumption and remain detectable for up to 99 hours[86]. They are also present in skin and hair and can be detected in the latter up to 2 months after abstinence[85]. They have been shown to be sensitive and specific at distinguishing social from heavy or alcohol dependent drinkers[87].

1.2.2.2 Indirect alcohol biomarkers

1.2.2.2.1 Routine laboratory tests

Alcohol can affect a number of routine laboratory tests that are often used as an adjunct to support a clinical suspicion of heavy alcohol intake[88]. Although most of these lack sensitivity and specificity, particularly in patients with CLD, γ-GT, MCV and the aminotransferases are still frequently used due to their low cost and availability.

- **Gamma-glutamyl transferase (γ-GT).** A cell membrane anchored enzyme present in several tissues (e.g. hepatocytes, biliary epithelial cells, renal tubules) that is involved in glutathione metabolism[89]. γ-GT levels rise after alcohol intake due to increase transcription of γ-GT genes and accelerated release from damaged or dead liver cells[90]. In contrast to hepatobiliary disease a rise in alkaline phosphatase (ALP) is not usually observed and a ratio of γ-GT to ALP >2.5 has been used to suggest alcohol induced acute hepatic damage[91]. Levels rise markedly only after at least 5 drinks/day for a minimum of 5 weeks and return to normal after 4-5 weeks of abstinence[92,93]. It has been demonstrated to have poor sensitivity, particularly in women and younger people (<30

years)[94,95]. It is also not specific, influenced by drugs (e.g. phenytoin, amiodarone, steroids[96]), obesity[97] and oxidative stress[98]. In fact, it is suggested as a marker for vascular disease, with elevations demonstrated in cardiovascular disease, stroke, type 2 diabetes and hypertension[99-102]. Its utility as an alcohol marker is therefore limited, but despite this it remains a commonly used marker of chronic heavy drinking due to its availability and low cost[103].

- **Mean Corpuscular Volume (MCV).** Macrocytosis is associated with chronic alcohol intake[104], in a dose dependent manner[105], and normalises slowly after 2-4 months of abstinence[106]. Although alcohol consumption less than 40g/day can increase MCV 1-2fL, at least 1 month of more than 60g/day is required to raise the MCV above the reference range[105,107]. It is suggested that the cut-off of red blood cell size should be ≥98fL rather than 95fL to improve specificity[105]. The exact mechanism remains unclear but may involve immunoglobulins binding to red cells[108] or a direct haemotoxic effect of ethanol and its metabolites[109]. It is best at detecting heavy drinking in adults aged 30-60 years but has been shown to have poor sensitivity (33%)[95]. It is also not specific since red blood cell size is affected by several conditions e.g. haematological disorders, B12 and/or folate deficiency and hypothyroidism. However, since the factors influencing MCV are less prevalent, macrocytosis is probably more reliable than γ-GT as a marker of heavy alcohol intake.
- **Aspartate and Alanine aminotransferases (AST and ALT)**. ALT and AST are enzymes involved in amino acid metabolism that are located mainly in the liver, although AST is also expressed in a number of other tissues. Long-term heavy alcohol consumption promotes mitochondrial AST translocation to the cell membrane and an upregulation in expression at the transcriptional level[110]. A subsequent rise in AST to ALT ratio above 2 is suggestive of alcohol as the aetiology[111,112]. However, amino-transferases mainly reflect liver damage and the AST to ALT ratio is also increased in cirrhosis. Furthermore, they can be affected by factors such as age, obesity, and medications, thus have low and variable sensitivity and specificity and are not recommended as biomarkers of heavy drinking[95].

1.2.2.2.2 Carbohydrate Deficient Transferrin (CDT)

The glycoprotein transferrin is synthesised in the liver and participates in iron transport. It consists of a single polypeptide chain and two N-linked glycan chains, which can be bi-, tri, and rarely tetra-antennary[113]. These contain terminal sialic residues, resulting in nine different glycoforms, namely asialo- to octasialo-transferrin (total of 0-8 sialic residues respectively)[113,114]. The relative amount of these glycoforms detectable in human serum varies, with tetrasialotransferrin being the most common glycoform (~75%) (Figure 1.3)[115].

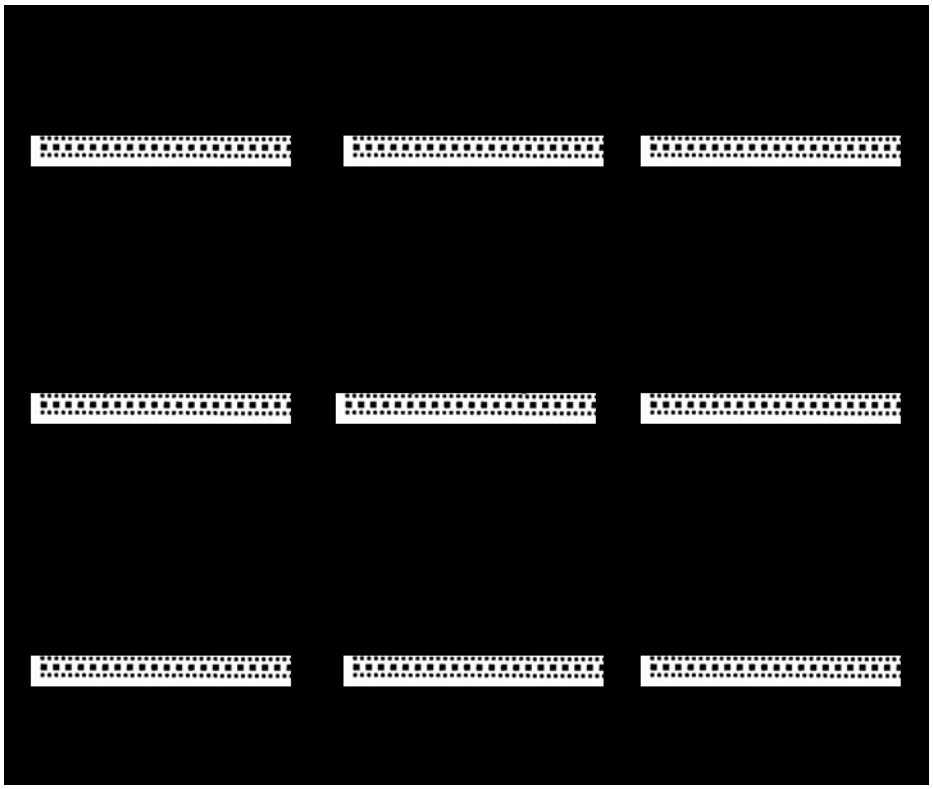


Figure 1.3. Transferrin glycoforms and relative amount (%) present in healthy human serum. ▭ Polypeptide chain; — Glycan chain; ● Sialic acid residue.

CDT refers to a temporary alteration in the glycosylation pattern of transferrin resulting in an increase in the relative amounts of disialo- and asialo-transferrin and a decrease in tetrasialotransferrin. It occurs as a result of sustained heavy alcohol consumption (50-80 g of alcohol/day for at least 2 weeks)[116]. Altered transferrin glycosylation patterns return to baseline levels within 2 to 5 weeks following complete abstinence from alcohol[117]. Using the standardized reference measurement technique with high performance liquid chromatography (HPLC) and quantification of disialotransferrin as a percentage of total transferrin (%CDT), a value of >1.7 is considered to be the most specific serum biomarker for sustained heavy alcohol consumption[118,119].

It has a high specificity with few circumstances associated with “false-positive” %CDT results using HPLC. These include genetic transferrin variants[115], rare congenital disorders of glycosylation[120] and pregnancy[121,122]. In contrast, the diagnostic sensitivity of %CDT for detection of heavy alcohol intake is low. Previous studies using older methods of CDT analysis such as immunoassays and anion-exchange methods have identified several patient characteristics that affect diagnostic sensitivity[123-128]. These characteristics include gender and metabolic risk factors such as obesity, insulin resistance, hypertension and dyslipidaemia. CDT was also found to be affected in patients with liver disease in the absence of alcohol abuse[129]. Many of these interferences were initially attributed to the older assay methods employed[130,131]. However, following the advent of the standardised HPLC technique there has been a report suggesting that body mass index (BMI) influenced %CDT, although not to a clinically relevant extent[118]. In addition, cirrhosis led to inaccurate quantification of %CDT due to poor chromatographic resolution of disialotransferrin from trisialotransferrin (di-tri bridging phenomenon)[132,133]. Hence the clinical utility of %CDT in patients with CLD is not clear and requires further investigation.

1.2.2.3 Biomarkers in combination

Due to the poor sensitivity and specificity of traditional biomarkers (γ-GT, MCV, AST and ALT) clinicians often interpret them together. Similarly several biomarker combinations have been proposed including traditional biomarkers and CDT[134-136]. The most common combination described includes CDT and γ-GT, which since first described[137] has evolved to a mathematically formulated equation (0.8*ln(γ-GT) + 1.3*(CDT)) that is elevated in a higher percentage of alcohol abusers than either CDT or γ-GT alone[138-140]. The performance of this formula reportedly improved with the addition of the newer %CDT assay, at that time[141], but has not been further evaluated since the introduction of the standardised HPLC method. Combinations have also included CDT, γ-GT, alcohol metabolites and screening instruments with good effect[142,143].

1.2.2.4 Limitations of alcohol biomarkers

The blood alcohol concentration obtained after consumption of a specific amount of alcohol varies between individuals as it is influenced by physiological processes that are affected by genetic and environmental factors[58]. The rate of alcohol absorption is altered by a number of factors e.g. activity of gastric alcohol dehydrogenase, gastric emptying rate, feeding state and meal composition, and amount and type of alcoholic drink consumed[144]. Furthermore the distribution of alcohol throughout the total body water, volume of distribution (Vd), varies between individuals and is determined largely by age, gender and weight. Levels of biomarkers can thus vary despite the same amount of alcohol consumed, making it difficult to predict the response of the biomarker to the drinking pattern.

Biomarkers can be influenced by factors independent of alcohol concentration e.g. liver disease (AST to ALT ratio is raised in cirrhosis) and cardiovascular disease (γ-GT elevated). Biomarkers can also respond differently between individuals e.g. both CDT and γ-GT have been demonstrated to increase at lower amounts of alcohol in subjects with a prior history of heavy alcohol intake[21,145].

Technical or laboratory issues can influence interpretation of results. The spectrum of these issues range from handling and processing of samples (e.g. haemolysed samples) to lack of standardisation of test results between laboratories (e.g. γ-GT). Furthermore techniques have changed over time, thus it is difficult to compare and interpret results using older analytical methods (e.g. CDT).

Studies into alcohol biomarkers also suffer from a lack of standardisation internationally regarding the amount of alcohol in a standard unit. This affects the already variable amount of alcohol (g/day or week) used to define alcohol groups e.g. “heavy drinker”, making it difficult to compare results between studies. Furthermore, the gold standard (Time line follow back alcohol history) has limitations and is not frequently used, so the comparator, is often a simple quantitative frequency history (e.g. AUDIT) that relies on the subject being able to recall and report honestly.

1.3 Assessment of hepatic fibrosis

Progressive accumulation of hepatic fibrosis is the common pathologic response of the liver to chronic injury, irrespective of the underlying aetiology. Its deposition leads to structural changes of the liver which disrupt the hepatic microcirculation and cellular physiology that eventually result in cirrhosis and its end-stage complications. Detection and quantification of hepatic fibrosis is important within the hepatology clinic to guide therapeutic decisions, determine prognosis and follow disease progression. However, there is also an increasing need for a pragmatic screening test in general clinical practice, to triage patients with CLD for referral and further investigation, particularly due to the rising prevalence of NAFLD.

1.3.1 Pathophysiology of hepatic fibrogenesis

Hepatic fibrogenesis is a wound healing response triggered by liver injury, and mediated by cross-talk between liver parenchymal and non-parenchymal cells, as well as infiltrating inflammatory cells. Progressive fibrosis results from excessive accumulation of extracellular matrix (ECM) components in the context of chronic injury, due to increased synthesis and decreased degradation of ECM (Figure 1.4) (reviewed in [146]). The main protagonist in this balance is the hepatic stellate cell that undergoes a phenotypic switch from a quiescent, vitamin A storing cell, into a proliferative, fibrogenic and contractile myofibroblast. These cells synthesise and secrete large amounts of fibril forming collagens, especially collagen type I and III, which over time replace type IV collagen and laminin that are the usual constituents of the sub-endothelial space of Disse. Hepatic stellate cells also express the components for matrix degradation, matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of MMPs (TIMPs), which are important regulatory molecules in tissue remodelling and repair and act by binding with MMPs.

1.3.2 Liver biopsy

Liver biopsy was introduced in the 1960s and significantly changed the field of hepatology[147]. It has since remained the “gold standard” for assessing hepatic fibrosis, and can provide additional information such as histological grading and support for aetiology of disease. However, the procedure is invasive, with associated morbidity (e.g. pain, bleeding, perforation of viscus) and mortality, poor patient acceptability and high cost. It is therefore not a suitable screening test and has limited use in monitoring disease progression[148-150]. There is also potential for sampling error, with only 1/50 000 of the liver being sampled in each biopsy, and it may be affected by interpretative error due to intra- and inter-observer variability of histological features[151-154]. To reduce this error, pathologists have tried to define the features of an adequate liver biopsy, which vary from >5 portal tracts and at least 15mm in length[155-157], to at least 11 portal tracts and 20-30mm in length[148,152].

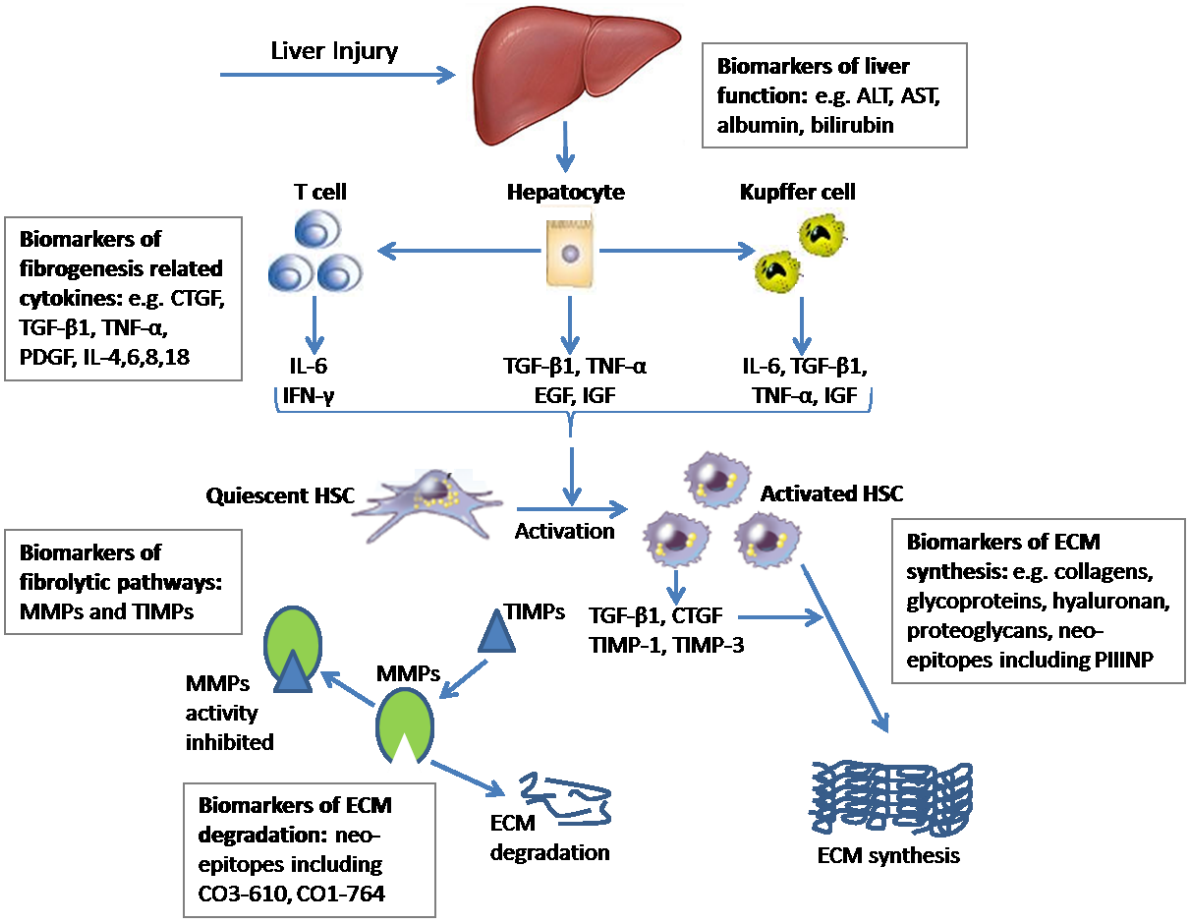


Figure 1.4. Molecular mechanisms of hepatic fibrogenesis and potential serum biomarkers. *Abbreviations: ECM, extracellular matrix; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PIIINP, N-terminal peptide of procollagen type III; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; HSC, hepatic stellate cell; CTGF, connective tissue growth factor; TGF-β1, tumour growth factor-β1; TNF, tumour necrosis factor; PDGF, platelet-derived growth factor; IL, interleukin; EGF, epidermal growth factor; IGF, insulin-like growth factor; IFN-γ, interferon-γ; CO3-610, collagen type III fragment generated by MMP-9; CO1-764, collagen type I fragment generated by MMP-2,9,13.* (Adapted from [158]).

1.3.3 Patterns of hepatic fibrosis and histological staging systems

The extent and distribution of hepatic fibrosis is an important marker of hepatic injury and differs between aetiologies of liver disease. Several histologic patterns have been characterised, which can occur singly or in combination. The following are the most common histological patterns of fibrosis:

- **Portal and periportal fibrosis.** Excess connective tissue forms within the portal tracts, which expand into the adjacent parenchyma. If the cause persists fibrous tissue occurs in the periportal region and may extend into the neighbouring parenchyma forming fibrous septa.

- **Subsinusoidal/perisinusoidal fibrosis.** Strands of connective tissue extend along the sinusoids to surround single or small groups of hepatocytes. Two types of subsinusoidal fibrosis (SSF) have been described: 1) coarse SSF, a chicken-wire or lattice-work appearance, with relatively coarse strands of collagen that are often asymmetrical and can form thickened fibrous septa[159]; 2) diffuse fine SSF, characterised by extensive, very fine fibrous bands, often single, symmetrically distributed throughout much or all of the lobule[160]. This fine SSF has infrequently been reported and is likely under recognised since it is not easily seen except on high power analysis following non-routine stains (e.g. Sirius red) (Figure 6.5).

- **Perivenular fibrosis.** Connective tissue is deposited in the centrilobular zone around the central vein. The amount of deposition varies from minor wall thickening to marked scarring of the centrilobular region. Fibrous septa can form as fibrosis spreads and connects with other centrilobular areas or with portal tracts.

- **Bridging/septal fibrosis.** Represents an extension of periportal or perivenular fibrosis and is a marker of progressive disease. Connective tissue septa extend across lobules and connect portal tracts and central veins. Shape and size of fibrous septa vary, ranging from slender, well defined bands to broad irregular collagenous zones that can encompass whole lobules. Bridging fibrosis can be separated into portal-portal, portal-central, and central-central types, based on the structures involved.

- **Cirrhosis.** A diffuse process characterised by a change from normal architecture to structurally abnormal nodules by annular fibrosis. There are 3 main categories: micronodular; macronodular and mixed. It can also be described by grading the severity, including: incomplete septal cirrhosis, very slender septa radiating from enlarged fields toward the center of the lobule; early cirrhosis, thin fibrous septa dissecting nodules; advanced cirrhosis, wide scars, which may contain large portal fields, and clusters of regenerative hepatocytes.

A number of histological staging systems have been proposed to assess the amount and distribution of hepatic fibrosis. Many of these systems were developed for specific diseases (e.g. Knodell, HBV[161]; METAVIR, HCV[162,163]), but they are broadly divided into portal based fibrosis (e.g. Knodell[161], METAVIR[163], Ishak[164]) and central based fibrosis (e.g. grading and staging of NASH[159] and the NASH clinical research network scoring system[165]). These systems are semiquantitative and vary in the number of stages used to score fibrosis (e.g. Knodell (n=4), METAVIR (n=5), Ishak (n=7)). Two of the most commonly used histological staging systems are METAVIR and Ishak (Table 1.5). These systems are portal based and do not apply to centrizonal liver disease and are thus limited in mixed aetiologies (e.g. HCV and alcohol). Subsequently studies with mixed aetiologies of liver disease may use a modified system to stage fibrosis (e.g. modified METAVIR score), which incorporate central fibrosis into the staging system.

Table 1.5. Staging systems for portal based fibrosis.

Histological description	Ishak	METAVIR
No fibrosis	0	0
Some fibrous portal expansion without septa	1-2	1
Fibrous expansion of most portal areas ± some septa	3	2
Fibrous portal expansion with marked bridging	4-5	3
Cirrhosis	6	4

1.3.4 Quantitative digital image analysis of liver fibrosis

Recently quantitative digital image analysis has been applied to assess liver fibrosis. It accurately calculates the proportion of collagen in an area of liver tissue, from a digital image of a liver section stained for collagen deposition (usually with Sirius red or trichome), by counting positive pixels. Unlike histological staging systems, collagen proportional area (CPA) is a continuous measure of the amount of collagen present and has less inter and intra-observer variability. As it measures all collagen, it measures features not incorporated into histological staging systems, such as SSF, and may thus have other prognostic implications. Studies have demonstrated that CPA is significantly correlated with histological stage, serum markers of fibrosis and clinical outcomes[166-169]. Importantly, this method has been demonstrated to be accurate and reproducible with small biopsy liver samples (5-10mm)[170].

1.3.5 Non-invasive assessment of hepatic fibrosis

Despite the recent advances of biopsy-based assessment of hepatic fibrosis, there remains an urgent need for an accurate non-invasive test to aid the assessment and management of patients with CLD. Over the last 15 years there has been substantial research to determine the molecular mechanisms that lead to liver fibrosis and cirrhosis and to evaluate the clinical and translational implications of a number of the variables identified, including the development of non-invasive biomarkers. There has also been substantial development in imaging techniques, particularly for the quantification of liver stiffness.

1.3.5.1 Serum biomarkers

Blood tests are generally acceptable since they are minimally invasive and have few complications. Serum biomarkers are thus potentially ideal for identifying and monitoring disease progression in patients with CLD. However, the tests need to be sensitive, specific, reproducible and cost effective, if they are to be used in clinical practice. A number of biomarkers have been identified (Figure 1.4 and Table 1.6), but the cost and technology involved and the lack of validation studies prohibits routine use for many of them. Two main groups of serum biomarkers of fibrosis have been described:

- **Indirect biomarkers.** Biochemical parameters that reflect functional alterations of the liver and include: markers of synthetic function (clotting factors, bilirubin, cholesterol and albumin); markers of hepatic inflammation (e.g. transaminases) and markers of processes that deregulate as liver fibrosis progresses and function deteriorates (e.g. platelet count and insulin). They are generally easy and inexpensive to measure and are often routinely performed in patients with CLDs.
- **Direct biomarkers.** Based on the pathophysiology of hepatic fibrogenesis and include profibrotic cytokines and ECM components, and thus give information on matrix turnover and activity of fibrogenesis. They are not routinely determined in clinical practice and are therefore less readily available and are often more expensive.

Table 1.6. Potential biomarkers of hepatic fibrosis (adapted from[171])

Indirect markers	
• Simple liver function tests	Albumin, aminotransferases (ALT, AST), bilirubin, γ-glutamyl transferase
• Haematological variables	Platelet count, prothrombin time
• Others	Apolipoprotein, cholesterol, globulins, glucose, insulin, haptoglobin
Direct markers	
• Collagen and ECM molecules and enzymes	N-terminal peptide of procollagen, laminin, TIMP-1, TIMP-2, hyaluronan, MMP-2, MMP-9, fibronectin, type IV collagen
• Cytokines	Platelet-derived growth factor, tumour growth factor-β1, angiotensin-II, connective tissue growth factor, tumour necrosis factor, IL-4,IL-6,IL-8,IL-18
• Proteomic markers	Microfibril-associated protein 4, tropomysin, galectin-3-binding protein
• Genetic markers	Single nuclear polymorphisms of AZIN1, TLR4, TRPM5, AQP2, STXBP5L

Table 1.6 notes. *Abbreviations: TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase; IL, interleukin; AZIN1, antizyme inhibitor 1; TLR4, toll-like receptor 4; TRPM5,* transient receptor potential cation channel subfamily M member 5; AQP2, aquaporin 2; STXBP5L, syntaxin binding protein 5-like.

Although both indirect biomarkers (platelet count and prothrombin time)[172,173] and direct biomarkers (hyaluronan (HA) and TIMP-1)[174,175] have been used on their own to predict fibrosis, they are more commonly used in combination to produce composite scores. A number of models have been described to predict fibrosis, which range from simple (e.g. AST to ALT ratio) to complex (e.g. Fibrotest) or are disease specific (e.g. NAFLD Fibrosis score). Most of them have good accuracy for advanced fibrosis, but not for mild/intermediate stages. Some commonly used scores are discussed below and their performance shown in Table 1.7.

Table 1.7. Diagnostic performance of serum biomarker panels in patients with CLD

SCORE	AUROC		Sensitivity		Specificity		NPV		PPV		References
	≥F2	F4	≥F2	F4	≥F2	F4	≥F2	F4	≥F2	F4	
AAR	N/A	0.51-0.83	N/A	46.7-78.0	N/A	95.9-100.0	N/A	80.7-89.0	N/A	73.7-100.0	[176,177]
APRI	0.69-0.88	0.61-0.94	41.0-91.0	57.0-89.0	47.0-95.0	70.9-95.0	64.0-86.0	93.0-98.0	21.8-88.0	38.0-57.0	[176-184]
FIB-4	0.82-0.89	0.79-0.91	37.6-74.3	72.7-85.0	80.1-87.5	65.0-88.7	74.2-94.7	95.0-96.9	75.6-82.1	40.4	[184-188]
Fibrotest	0.74-0.87	0.71-0.87	58.9-77.0	50.0-87.0	72.0-98.0	70.0-92.9	66.7-81.0	44.0-97.0	59.6-90.0	49.1-93.0	[181-184,189-193]
Hepascore	0.79-0.85	0.85-0.94	50.5-82.0	71.0-87.0	65.0-92.0	84.0-98.8	63.5-78.0	89.6-98.5	70.0-88.0	45.4-64.9	[182,184,194,195]
ELF test	0.78-0.98	0.86-0.92	70.0-94.0	62.0-91.0	31.0-93.0	59.0-89.0	65.0-92.0	95.0-98.0	27.5-88.0	29.0-73.0	[181,193,196-202]

Table 1.7 notes. *Abbreviations: AUROC, area under the receiver operator characteristic curve; NPV, negative predictive value; PPV, positive predictive value; AAR, AST to ALT ratio; APRI, AST to platelet ratio index; ELF, Enhanced Liver Fibrosis.*

- **AST to ALT ratio.** One of the first combinations to be used to stage liver fibrosis, with a ratio >1 being indicative of cirrhosis[203]. It reflects the reduced clearance of AST by the sinusoidal network in patients with liver fibrosis[204]. It is also likely contributed to by the increased mitochondrial injury in advanced fibrosis, resulting in a more marked release of AST relative to ALT[205,206].
- **AST to platelet ratio index (APRI).** A simple score, which is calculated using the equation: *((AST [U/L] / upper limit of normal AST [U/L]) / platelet count [10^9/L]) x 100*[180]. It is one of the most investigated non-invasive markers of liver fibrosis, which can rule out or rule in both significant fibrosis (cut-off 0.5 and 1.5 respectively) and cirrhosis (cut-off 1 and 2 respectively). However, APRI's major role appears to be the exclusion of significant fibrosis or cirrhosis[178].
- **FIB-4.** A simple algorithm which is calculated using the equation*: (age [years] x AST [U/L]) / (platelet count [10^9/L] x √ALT [U/L])*[207]. FIB-4 was developed as a non-invasive panel to stage liver disease in subjects with HIV/HCV co-infection[207] and has since been validated in patients with HCV monoinfection[185], HBV[187] and NAFLD[188]. It is good at excluding or confirming advanced fibrosis (cut-off 1.45 and 3.25 respectively), but a substantial proportion of patients are unclassified by the test.
- **Fibrotest.** Consists of a panel of five biomarkers that are adjusted to the patient age and gender using the complex algorithm: *4467 x log (alpha2-macroglobulin [g/L]) – 1357 x log (haptoglobin [g/L]) + 0.0821 x (age [years]) + 1737 x log (bilirubin [µmol/L]) – 1184 x (apolipoprotein A1 [g/L]) + 0.301 x gender (male=1, female=0) – 5.054*[189]. Fibrotest is the most studied indirect serum biomarker panel test and has been applied to various aetiologies of CLDs, including HCV[192,208-210], HBV[211,212], HIV/HCV[213], NAFLD[184,214] and alcohol related liver disease (ALD)[215]. The test is good at the extreme stages of fibrosis, but is indeterminate in the intermediate ranges[191]. It has recently been validated for use during follow-up to monitor disease progression for the most frequent CLDs[216].

- **Hepascore.** A complex score calculated using the following equation: *y / 1 + y, when y = exp [–4.185818 – (0.0249 x age[years]) + (0.7464 x gender (male=1, female=0)) + (1.0039 x α2-macroglobulin [g/L]) + (0.0302 x hyaluronic acid [µg/L]) + (0.0691 x bilirubin [µmol/L]) – (0.0012 x GGT [U/L])]* [194]. It was developed in a cohort of patients with HCV[194] and has since been validated in NAFLD[184], ALD[217] and HBV[218]. A score ≥0.5 indicated significant fibrosis, a score <0.5 excluded advanced fibrosis and a cut-off of 0.84 was used to predict cirrhosis. It had good performance, but it also has significant overlap between patients with mild and moderate fibrosis.
- **Enhanced Liver Fibrosis test (ELF).** A simplified version of the original European liver fibrosis (OELF) panel, which included age in the algorithm. The OELF was originally developed from evaluating algorithms combining up to 9 surrogate markers of liver fibrosis for their ability to discriminate between biopsy proven liver fibrosis in 921 subjects[196]. The final ELF algorithm is based on the measurement of 3 circulating direct serum markers of liver matrix remodelling; hyaluronan (HA), N-terminal peptide of procollagen type III (PIIINP) and tissue inhibitor of metalloproteinase-1 (TIMP-1). It is a standardised method and the ELF score can be autocalculated by the ADVIA Centaur XP system using the equation: $2.278 + 0.851\ ln(C_{HA}) + 0.751\ ln(C_{PIIINP}) + 0.394\ ln(C_{TIMP\text{-}1})$. The manufacturer stated a lower cut-off value of 7.7 and an upper cut-off value of 9.8, which were derived using a sensitivity of about 90% to discriminate Ishak stages 0 to 2 from 3 to 6 and a specificity of 90% to discriminate 0 to 4 from 5 to 6, respectively, in a cohort of 921 patients. However many studies have derived their own cut-off based on their individual patient cohort. The ELF test has been validated in patients with mixed aetiologies of CLD[197,219], HCV[199,201], HBV[202], NAFLD[198] and demonstrated to perform well in ALD[196]. It has been shown to reliably exclude or detect significant fibrosis in patients with CLD and is a good diagnostic tool in clinical practice for identifying cirrhosis[196,220]. It has also been demonstrated to predict clinical outcomes (liver related morbidity and mortality), with a unit change in ELF score associated with a doubling of risk of liver related outcome[221].

1.3.5.1.1 Limitations of serum biomarkers of liver fibrosis

Although serum biomarkers have clear benefits they also suffer limitations. Sample collection and storage can significantly influence results, e.g. postprandial state (HA)[222] and haemolysed samples (e.g. PIIINP[223], AST[224]). Non-standardisation of assays and lack of agreement on the upper limits of normal can lead to variability[225]. Patient demographic and clinical variables may impact results (e.g. smoking[226], alcohol[227], ethnicity[228], age[201], gender[201], BMI[201]). There is also a lack of liver specificity with many tests, e.g. HA can be affected by renal failure[229,230] or extrahepatic fibrogenesis (e.g. rheumatoid arthritis[231], psoriasis[232], scleroderma[233]) and bilirubin is elevated in haemolysis, Gilbert's syndrome, or biliary obstruction. Furthermore, systemic inflammation from any cause may produce false positive results in acute phase reactants, such as HA, α2macroglubulin, platelet count and PIIINP. Acute hepatitis can also cause marked derangement of tests (e.g. ALT, AST). The clear disadvantage of serum biomarkers is their poor ability to differentiate intermediate stages of fibrosis, compared to cirrhosis. This is likely due to the relatively higher influence of inflammation on fibrosis markers in early fibrosis, when the amount of deposited matrix is less.

Complex serum panels are more accurate than simple models[184,197], and many of the complex panels are better standardised, with acceptable inter-laboratory reproducibility for clinical practice[234,235]. However, they are more costly, less widely available and some are more difficult to calculate. This is important, particularly if the test is to be used for screening.

1.3.5.2 Imaging techniques

Routine imaging modalities (e.g. ultrasound, computed tomography and magnetic resonance imaging (MRI)) can identify cirrhosis if signs of portal hypertension and/or a nodular or irregular liver surface are present. However, these features are often absent in patients with milder degrees of fibrosis or less advanced cirrhosis. Further developments of MRI may be helpful in the assessment of hepatic fibrosis, namely diffusion weighted MRI and MR spectroscopy. However, both of these are currently poorly accessible due to the need for an MRI scanner and require further refinement before they can be considered for clinical use[236,237]. Currently routine liver imaging therefore does not have adequate sensitivity to be used as a non-invasive test for fibrosis.

The deposition of fibrotic tissue in the liver changes its physical properties or stiffness, which has been exploited by non-invasive imaging techniques. Three of the main techniques are described below:

- **Transient elastography (TE).** First described in 2003, TE measures the liver stiffness using a transducer probe mounted on a vibrating axis[238]. Mild amplitude, low frequency (50Hz) vibrations generate 1-dimensional mechanical waves that are then followed by pulse echo ultrasound, measuring the shear wave velocity. The resulting liver stiffness measurement, measured in kiloPascals, correlates with the degree of hepatic fibrosis, with higher liver stiffness measurements reflecting a faster wave velocity and thus a higher degree of fibrosis.

  TE is increasingly used in hepatology centres and offers a simple, safe and efficient way to estimate hepatic fibrosis[239]. The majority of studies have occurred in patients with HCV and have demonstrated that TE is effective for detecting cirrhosis, but less accurate at identifying milder stages of fibrosis[240-242]. It is affected by liver inflammation, resulting in overestimation of liver stiffness up to 3 fold[243-245], and a significant correlation has also been demonstrated with histological steatosis[219]. Liver architecture, which varies between different liver diseases, has also been shown to influence TE and has been particularly evident in HBV cohorts, where macronodular cirrhosis is more common and can result in

an underestimate of liver stiffness[246]. Cholestasis (e.g. biliary obstruction), mass lesions within the liver (e.g. tumour) and liver congestion (e.g. heart failure) have also been shown to cause overestimation of liver stiffness[239].

TE is attractive as it is painless, quick, reproducible and is not typically affected by extrahepatic disorders. However, it has been demonstrated that accurate readings were not obtainable in about 20% of patients mainly due to obesity[247]. The recent introduction of the Fibroscan® XL probe has enabled measurement of liver stiffness in significantly more obese patients than the M probe, but currently it is less accurate and new cut-off values need to be validated[248].

- **Magnetic resonance elastography (MRE).** This uses a pneumatic driver that is placed against the anterior abdominal wall and vibrates at low frequencies (40-120 Hz) to generate a mechanical wave formation which propagates into the liver. An MRI sequence then images the propagating waves and enables the liver stiffness to be measured. Promising results have been demonstrated, particularly in the diagnosis of cirrhosis[249-251]. The main advantages of MRE are that it is operator independent, can be performed on obese patients (as long as they can fit into the magnet bore) and large cross-sectional areas of hepatic parenchyma can be evaluated. It is however limited by the standard contraindications for MRI (e.g. pacemaker/defibrillator, aneurysm clip, claustrophobia), as well as pathologic processes described for TE.
- **Acoustic radiation force impulse (ARFI) imaging.** This uses short-duration acoustic pulses to generate shear waves which are then tracked by ultrasound. An initial pilot study demonstrated a similar performance to TE, although 2% of patients were excluded from the study due to failed TE (secondary to obesity)[252], and a subsequent study confirmed the usefulness of ARFI imaging for the assessment of liver stiffness [253]. The main advantage of this technique is that it is integrated into a conventional ultrasound system, thus it can be performed at the same time as liver ultrasound.

1.3.5.3 Sequential algorithms

A limitation for studies investigating non-invasive biomarkers of hepatic fibrosis is that the "gold standard", liver biopsy, is not 100% accurate in its estimation of fibrosis, largely due to sampling error[152]. Therefore even if the biopsy was 90% sensitive and specific, which many are not[152], a perfect biomarker could only obtain an AUROC of 0.9. However, despite this many biomarkers have only a 75-80% diagnostic accuracy, and thus are considered inadequate for clinical practice[155].

Combinations of serum panels have been suggested to improve sensitivity and reduce the number of liver biopsies performed to stage fibrosis. Some are also cost efficient, as they often employ a simple cheap test first. An example is the Sequential Algorithms for Fibrosis Evaluation (SAFE) which was proposed in 2006 for use in patients with HCV. The model used APRI first, since it is simple and cheap, then Fibrotest, which is more expensive and complex. Using this algorithm they attained a >94% diagnostic accuracy for advanced fibrosis and would have reduced the requirement for liver biopsy by 60-70%[183]. Further validation of this algorithm achieved diagnostic accuracies of 90% and 93% for advanced fibrosis and cirrhosis, respectively[254]. Similar studies have been performed using other panel combinations with comparable diagnostic accuracies[255-258].

Serum panels have also been combined with imaging modalities, mainly TE. An example is the Castéra algorithm, which combined Fibrotest and TE (Fibroscan®) in patients with HCV. The algorithm had an excellent performance for detecting both significant fibrosis and cirrhosis (accuracy >90%), and could have reduced the requirement for liver biopsy[208]. Further studies have also demonstrated increased accuracy with combinations than the tests alone, in cohorts with mixed CLD[259] and HBV[260].

1.3.5.4 Future developments

Proteomic studies have identified a number of novel proteins associated with fibrosis that have been used to successfully predict fibrosis in patients with HCV, HBV and NAFLD[261-263]. Currently these methods are not suitable for clinical use, but may identify other biomarkers for further assessment in the research setting[264].

Development of methodologies for assessment of genetic data has enabled detection of single nucleotide polymorphisms (SNPs) in specific genes that are linked with liver fibrosis[265,266]. However, initial genetic scores have not surpassed the accuracy of current non-invasive biomarkers[267]. Prospectively they may be useful in predicting patients likely to develop liver disease or its complications, particularly hepatocellular cancer[268].

Isotope labelled breath tests are being increasingly validated in the management of patients with liver disease. The ^{13}C-caffeine breath test exploits the extensive hepatic metabolism caffeine undergoes, and thus patients with liver disease are likely to have reduced caffeine metabolism, hence lower levels of $^{13}CO_2$ compared to healthy controls. It has been demonstrated to differentiate significant fibrosis[269] and cirrhosis[270]. Similarly, methacetin is metabolised by the healthy liver to acetaminophen and CO_2. The ^{13}C-methacetin breath test has been demonstrated to have AUROCs of 0.83 and 0.96 for identifying advanced fibrosis and cirrhosis, respectively[271], and have been used to determine suitability for hepatic resection and to potentially aid prioritisation of patients for transplant and early recognition of complications post liver transplantation[272,273].

1.4 Biomarkers in the assessment of ascites

Regardless of aetiology, the majority of morbidity and mortality associated with CLD occurs among people with advanced fibrosis, who are at risk of developing hepatocellular carcinoma or complications of end-stage liver disease (ascites, hepatic encephalopathy, variceal haemorrhage). The development of cirrhosis is associated with a marked increase in hospital admissions and health care costs[274]. This health care burden is expected to escalate as more patients present with cirrhosis, due to poor uptake of HCV therapy, and increasing rates of alcohol abuse and obesity[275].

Of the complications of end-stage liver disease the development of ascites is especially important, as it heralds the onset of decompensation and a change in survival from 80% at 5 years to 50% at 5 years[276]. Patients with cirrhosis and ascites have recurrent hospital admissions, often within a month of a previous discharge that could potentially be prevented with better coordination of care. The identification of clinical parameters associated with development or recurrence of complications (e.g. SBP) and hospital admissions is likely to enable an improved approach to hospital use, leading to better patient management and clinical outcomes and subsequent cost savings. In addition, increased understanding of the processes involved in the development of ascites and SBP may provide biomarkers and/or targets for therapy.

1.4.1 Pathophysiology of ascites

Ascites is the pathologic accumulation of fluid within the peritoneal cavity, which has been described since ancient Egyptian times[277]. Although cirrhosis accounts for approximately 85% of cases[278,279], other liver pathology or non-hepatic disorders may also lead to its formation (Table 1.8). The pathogenesis of ascites differs between these aetiologies and can be used to help determine the underlying cause. The serum-ascites albumin gradient (SAAG) is widely used to differentiate disease processes due to portal hypertension from non-portal hypertensive causes (Table 1.8)[280].

Table 1.8. Causes of ascites according to the serum-ascites albumin gradient (SAAG) of ascites[280].

SAAG ≥11.1 g/L	SAAG <11.1g/L
Cirrhosis	Peritoneal carcinomatosis
Alcoholic hepatitis	Peritoneal tuberculosis
Congestive heart failure	Pancreatitis
Massive hepatic metastases	Serositis
Vascular occlusion	Nephrotic syndrome
Fatty liver disease of pregnancy	Bowel obstruction/infarction/perforation
Myxoedema	

In cirrhosis, the pathogenic processes that lead to ascites formation are still not completely understood. However, current evidence suggests that the development of cirrhosis and portal hypertension result in a backflow and stasis of vasodilatory substances (e.g. nitric oxide (NO))[281]. Their accumulation results in vasodilatation in the systemic and splanchnic circulations, but vasoconstriction in the renal circulation as it responds to the resultant hypoperfusion. This vasoconstriction occurs via the renin angiotensin aldosterone system, which is activated and leads to a gradual increase in renal sodium and fluid retention[281,282]. The excess retained blood volume likely then filters across into the peritoneal cavity, due to increased hydrostatic pressure and vascular wall permeability, and reduced oncotic pressure secondary to hypoalbuminaemia. Ascites develops as the reabsorptive capacity of the peritoneal surface and lymphatic system become overwhelmed[281,282].

1.4.2 Spontaneous bacterial peritonitis (SBP)

Bacterial infections remain a leading cause of morbidity and mortality for patients with cirrhosis and ascites. One of the most common bacterial infections, occurring in 10-25% of patients[283], is SBP. It is defined by an elevated ascitic fluid neutrophil count ≥250/mm^3 and is associated with a 20 to 40% in-hospital mortality[284]. Currently bacteria and/or their products (e.g. lipopolysaccharides, bacterial DNA) are thought to cross the intestinal wall, in a

process called bacterial translocation (BT), and then disseminate into the systemic circulation, via the mesenteric lymph nodes, eventually reaching the ascitic fluid[285]. SBP may result once bacteria overcome the patient's innate antimicrobial defences[286,287]. The main mechanisms thought to facilitate BT are: increased intestinal permeability, intestinal bacterial overgrowth and impaired local and systemic immunity[288,289].

1.4.2.1 Intestinal permeability

A single layer of epithelial cells acts as a barrier to prevent permeation of microorganisms and potentially harmful substances and selective permeation of others (e.g. nutrients)[290,291]. The intestinal barrier includes mechanical and secretory components, both of which are affected in patients with cirrhosis.

Mechanical components: Tight junctions are integral to the mechanical barrier function of the epithelial cells, which restrict paracellular movement of very small (2kDa) molecules such as bacteria or lipopolysaccharide. Studies have demonstrated that vital components of tight junction proteins have altered expression in cirrhosis, with reduced expression of occludin and claudin-1[292], and increased expression of the pore forming protein, claudin-2[293]. It has been postulated that the down regulation may result from cytokines such as tumour necrosis factor (TNF) and interferon gamma (IFN-γ), which are increased in cirrhosis[294], or when epithelial cells are under stress[295-297]. Interestingly, the decrease in intestinal tight junction proteins expression was profoundly associated with the presence of ascites and inversely correlated with clinical markers of raised portal hypertension[292]. Portal hypertension is associated with higher levels of NO, which can directly dilate tight junctions, inhibit ATP formation and hence increase intestinal permeability[298,299]. Furthermore portal hypertension may affect the integrity of the intestinal barrier by causing congestion and oedema of the bowel wall, with dilatation of the intercellular spaces[300,301].

Secretory component: This helps provide an impermeable barrier that can monitor and regulate bacterial attachment and infiltration into the host and includes mucins, bile salts, immunoglobulin A (IgA) and antimicrobial peptides (AMPs):

- Mucins create a layer of glycoproteins that protect the microvillus from direct contact with bacteria[302]. It provides two layers: 1) a firm inner layer that traps immune exclusion molecules[303] and is thought to therefore to be sterile[304]; 2) a loose outer layer that contains the commensal bacteria, which use it as a source of carbon[305]. Cirrhosis has been associated with a thickening of the mucus, which may contribute to bacterial overgrowth[306] and changes in the flora[307].
- Bile salts act as a detergent, neutralizing toxins, decreasing bacterial internalization and inhibiting small intestinal bacterial overgrowth (SIBO)[308,309]. They can also impact intestinal immunity[310]. Cirrhosis is associated with impaired secretion and increased deconjugation of bile salts, which may thus facilitate BT[311].
- IgA is the dominant immunoglobulin isotype in humans and plays an important role in humoral mucosal immunity, particularly in the gut lumen, which has 2-5g IgA secreted into it on a daily basis [312]. IgA antibodies primarily act by preventing attachment of pathogens to epithelial receptors, whilst agglutinating them in the mucus layer, preventing colonization. It has long been realised that serum IgA levels are raised in patients with alcohol related liver disease[313,314] and more recently NASH[315,316], and that they appear to increase with liver fibrosis[313,315]. Interestingly, decreased secretion of mucosal IgA into the gut lumen and reduced faecal IgA concentrations have been found in patients with cirrhosis[317], providing a plausible explanation for the development of SIBO and BT in these patients.
- AMPs are integral regulators of the intestinal microbiota composition and growth[318,319], and include defensins (α and β), cathelicidin and lysozyme, secreted phospholipase A[320]. The defensins appear to be the most important AMPs, but particularly the α-defensins[321], which are expressed predominantly by neutrophils and Paneth cells. The latter are

located at the base of the crypts of Lieberkühn and in addition to α-defensins also produce other AMPs. In cirrhosis it is suggested that there is a relative deficiency of Paneth cells, perhaps secondary to zinc deficiency[322], resulting in a decreased AMP secretion in response to microbial contact.

1.4.2.2 Intestinal bacterial overgrowth

The intestinal microflora is a dynamic mixture of microbes and in humans includes up to 40,000 species, although it is estimated 30-40 species account for 98-99% of the microbiota in healthy individuals[323]. The microbial density is usually sparse in the proximal small intestine, but sharply increases distally: jejunum (10^5 colony forming units (CFU)/ml)); distal ileum and caecum (10^8 CFU/ml); colon (10^{12} CFU/ml)[324]. SIBO, defined as >10^5 CFU/ml and/or the presence of colonic bacteria (e.g. *Enterobacter, E. coli, Bacteroides, Clostridium* and *Klebsiella*) in the upper jejunal aspirate[325], is common in patients with cirrhosis[326-329] and appears to be linked to liver disease severity[330]. Its development in patients with cirrhosis is likely multifactorial, including changes to the intestinal barrier and delayed small bowel transit, secondary to an overactive sympathetic nervous system[331,332]. Medications may also contribute to (e.g. proton pump inhibitors[333]) or prevent (e.g. cisapride[334]) development of SIBO.

In patients with cirrhosis the gut microbiota is documented to change from the dominance of Bacteroidetes and Fusobacteria seen in healthy individuals[323] to a relative abundance of Proteobacteria and Fusobacteria[335] and a decrease of Bacteroidetes[336-338]. These changes are associated with the development of a pro-inflammatory profile[335], which has been linked to hepatic encephalopathy[307]. SIBO is also more common in patients with previous hepatic encephalopathy and/or SBP[339,340]. This is likely contributed to by the overgrowth of potentially pathogenic bacteria (e.g. *E.coli, P. aeruginosa*, enterococci and streptococci) that are more adept at translocating across the gut lumen[336].

1.4.2.3 Impaired local and systemic immunity

In healthy individuals, there is a constant translocation of small amounts of bacteria and/or their products across the intestinal wall that then reach the portal circulation[341]. The innate immune system acts as the first line of defence against translocating bacteria by recognizing highly conserved pathogen-associated molecular patterns (e.g. lipopolysaccharide, muramyl dipeptide, bacterial DNA) via Toll-like receptors (e.g. TLR2, TLR4, TLR9) or NOD-like receptors (e.g. NOD2). The innate immune response includes phagocytic cells (e.g. neutrophils, monocytes, circulating and resident macrophages (Kupffer cells)), opsonins (e.g. immunoglobulins, complement), non-specific T cells and natural killer cells. The liver itself therefore plays a vital role in the innate immune response as it contains the major proportion of human reticuloendothelial cells[342] and is the first organ exposed to blood from the hepatic portal system. It has a key role in preventing bacteria and/or their products from reaching the systemic circulation.

In patients with cirrhosis, immune dysfunction contributes to pathological BT as bacterial translocation is no longer controlled. Impaired liver function results in reduced protein synthesis, with subsequent decreased opsonisation and dysfunctional phagocytic activity[343,344]. Intra and extrahepatic shunts result in blood being diverted away from the reticuloendothelial system, particularly Kupffer cells, which in addition to the reduced hepatocyte dysfunction, causes decreased clearance of lipopolysaccharide[345,346]. Subsequently there is a switch from a local hepatic immune system to a systemic pro-inflammatory state, which further impairs liver function and hepatic architecture[347,348].

Lipopolysaccharide has been demonstrated to lead to augmented expression of class II major histocompatibility complex and CD80 co-stimulatory molecule on the surface of monocytes[349], contributing to the inflammatory state in cirrhosis[350], with increased levels of TNF and interleukin (IL)-6[351]. However, prolonged exposure to lipopolysaccharide may cause a lipopolysaccharide tolerant phenotype with decreased human leukocyte antigen (HLA)-DR expression[352] and increased risk of sepsis related mortality[353]. Lipopolysaccharide can both prime[354] and activate[355] neutrophils, which can

therefore become fully activated in the systemic circulation, releasing pro-inflammatory cytokines and reactive oxygen species. Chronic activation of neutrophils may however lead to dysfunctional phagocytosis[356,357]. Insulin resistance, common in cirrhotic patients[358], may also be associated with impaired neutrophil chemotaxis/recruitment and dysfunction[359].

Importantly it has been demonstrated that not only lipopolysaccharides are responsible for immune dysfunction in cirrhotic patients, but that other bacterial products (e.g. peptidoglycans) may play a role. This is supported by the finding that polymorphisms in the TLR2 gene, the product of which (TLR2) recognises products of Gram-positive bacteria, are associated with increased susceptibility to SBP[360], in addition to NOD2[361,362] and TLR4[363]. Furthermore, bacterial DNA contains immunostimulatory unmethylated cytosine-guanosine dinucleotides (CpGs) that interact with TLR9 and increase levels of activated NF-κB in the absence of lipopolysaccharide[364]. The innate immune system is known to direct the adaptive immune responses and these interactions between CpGs and TLR9 have been reported to effectively bridge the innate and the adaptive immune response[365], which becomes attenuated in cirrhosis[351]. Cirrhotic patients have been reported to have decreased memory B cells and hyporesponsiveness to TLR9 stimulation[366].

1.4.3 Laboratory tests in the routine management of patients with ascites

Diagnostic paracentesis is performed under sterile conditions in patients with new-onset ascites or whenever SBP is suspected. Routine ascitic fluid analysis includes cell count and differential, albumin and total protein levels and ascitic fluid culture. Other ascitic fluid tests (e.g. lactate dehydrogenase, amylase, glucose, triglycerides and cytology) may be requested depending on the clinical situation or appearance of ascites. Routine blood tests are important in assessment of the patient, particularly to ensure that paracentesis will be safe[367]. In patients with suspected SBP, blood and urine cultures are also obtained.

1.4.3.1 Assessment of ascitic fluid

The normal appearance of ascites is clear to yellow and transparent; blood stained ascites is associated with malignancy or a recent paracentesis or invasive procedure (e.g. liver biopsy); very brown fluid can occur with bowel or biliary perforation; chylous ascites suggests injured lymphatic ducts, which causes a high triglyceride count (>100-200mg/dL)[368]. Cirrhosis is the most common non-surgical cause of chylous ascites[369]. In cirrhosis, the SAAG is elevated (>11.1g/L) from hepatic sinusoidal hypertension[370] secondary to capillarisation of the sinusoids, resulting in a low total ascites protein[371]. This can therefore be differentiated from other common causes of ascites: peritoneal involvement (e.g. malignancy, pancreatitis), low SAAG and high ascites total protein (>25g/L); postsinusoidal or posthepatic sinusoidal hypertension, high SAAG and high ascites total protein. Clinical history and examination are clearly important and other tests can be considered if indicated (e.g. pancreatitis, amylase; bowel/biliary perforation, bilirubin or Runyon's criteria[372]; malignancy, cytology; tuberculosis, stain for tubercle bacilli).

An ascitic fluid neutrophil count >500mm^3 is the single best predictor of SBP, however a neutrophil count ≥250/mm^3 is more sensitive and only slightly less specific, hence is used to diagnose SBP[373]. Detection of bacteria by inoculating blood culture bottles with ascitic fluid at the bedside is superior to classical culture techniques, but is still often negative despite neutrocytic samples[374]. Patients with a neutrophil count ≥250/mm^3 but negative ascitic fluid culture (culture-negative neutrocytic ascites) are thought to represent SBP with organisms refractory to culture or at relatively low concentration, since patient symptoms, signs and outcomes are similar to culture positive SBP[375]. Occasionally the ascitic culture is positive but the patient has a negative ascitic neutrophil count (<250/mm^3) (non-neutrocytic bacterascites) which can result from contamination but may represent an early form of SBP[376].

1.4.3.2 Identifying patients requiring primary prophylaxis for SBP

SBP is associated with significant mortality[284,377,378], but long-term antibiotic prophylaxis can result in development of resistant bacteria[379] and a higher than expected incidence of infections due to Gram-positive organisms[380]. Prophylaxis is hence only recommended for those at highest risk, which includes patients with cirrhosis and gastrointestinal bleeding and those with previous SBP[381]. It is also recommended in patients with cirrhosis and ascites with low ascites total protein (<15g/L), with evidence of renal impairment (creatinine ≥106μmol/L, blood urea nitrogen level ≥8.9mmol/L, or a serum sodium ≤130mmol/L) or liver failure (Child-Turcotte-Pugh (CTP) score ≥9 and a bilirubin ≥51μmol/L)[381]. Antibiotic prophylaxis is also suggested in patients with cirrhosis hospitalised for other reasons if the ascitic total protein is <10g/L[381].

1.4.3.3 Predicting clinical outcomes

Liver disease severity is used to predict clinical outcomes for patients with cirrhosis. Two main scoring systems are used: CTP, uses blood tests (bilirubin, albumin and international normalised ratio of the prothrombin time (INR)) combined with the presence and severity of ascites and encephalopathy[382]; model for end-stage liver disease (MELD), uses blood tests (bilirubin, creatinine and INR) according to a formula[383]. Both scoring systems can be used to predict survival and prioritise for liver transplantation[384,385], although the MELD score is regarded to be more accurate[386]. Interestingly, MELD can reportedly predict early readmission following hospital discharge for patients with decompensated cirrhosis[387,388]. This likely reflects the fact that MELD incorporates deterioration in both liver and renal function.

Renal dysfunction is very common in decompensated cirrhotic patients and is likely multifactorial in origin[389,390], including increased permeability of the intestinal barrier[391]. It occurs in about a third of patients with SBP, even in the absence of shock[392], and likely represents further splanchnic and systemic vasodilatation on the background of a lower baseline cardiac output that has the incapacity to increase[393]. Accordingly, NO, an important regulator of vascular tone[394] that is increased in patients with cirrhosis (particularly those with ascites[395]), has been demonstrated to be an independent predictor for the

development of renal impairment in patients with cirrhosis and SBP[396]. Markers of renal failure have also been shown to predict poor outcomes, including hospital readmission[397] and death[398-401], particularly in patients with SBP[402].

Cirrhosis is an independent risk factor for bacterial infections[403,404], which are linked to increased hospital admissions, prolonged hospital stays and worse outcomes[405,406]. Diagnosis and effective treatment are therefore paramount, but prediction and prevention of infections are preferable. Although the definitions of sepsis and systemic inflammatory response syndrome (SIRS)[407] are not fully applicable to patients with cirrhosis[408-410], they can help differentiate those with or without a bacterial infection[411,412] and predict mortality[412]. SIRS has been linked to prognosis in patients with cirrhosis even in the absence of overt infection, which may occur due to increased permeability of the intestinal barrier[412]. Biomarkers of inflammation are widely used in clinical practice to aid early diagnosis of infection, and their utility demonstrated in patients with cirrhosis, despite the majority being synthesised by the liver[413]. C-reactive protein (CRP) and procalcitonin (PCT) are reportedly the most reliable markers of inflammation, however many of the studies investigating their utility have used variable cut-offs, often at higher levels than normal[414,415]. PCT has been demonstrated to be superior to CRP as well as IL-6 and TNF[416,417], although CRP has been suggested to be a reliable and cheaper alternative[418]. Elevated CRP level has also been shown to predict development of infections[418], although the accuracy of CRP decreases in advanced liver disease and in the presence of ascites[418].

Due to the inability of established indicators of inflammation to identify or predict patients with or at risk of infection in patients with cirrhosis, alternative biomarkers are being investigated, including soluble urokinase plasminogen activator receptor[347], lipopolysaccharide-binding protein[419] and polymorphisms of TLR and NOD2[360]. However one of the most promising and frequently studied potential biomarkers is bacterial DNA. It can be detected in the blood and ascitic fluid of patients with advanced cirrhosis[420], and can identify patients prior to fully developed infection and/or poor outcomes[421].

1.4.4 Bacterial DNA

The development of nucleic acid amplification has revolutionised infectious disease diagnostics as it enables rapid culture-independent detection and identification of bacteria[422]. These techniques have also dramatically changed our understanding of microbial diversity particularly within the endogenous human microbiome[423,424], which may contribute to development of disease[425]. Targeting the16S ribosomal RNA (rRNA) gene allows both broad-range detection of most eubacterial species due to the highly conserved sequences of the gene and species level identification by exploiting the nine hypervariable regions (V1-V9)[426]. Although there has been some debate about which hypervariable region to target, the V3 and especially the V4[427] region have been suggested to be the most effective, particularly for sequencing platforms that produce shorter reads (e.g. Illumina (MiSeq))[428]. Furthermore, the V4 region has been documented to be the most robust hypervariable region for classifying gastrointestinal communities[429].

Detection of bacterial DNA in the serum and ascitic fluid of patients with culture negative non-neutrocytic ascites has been interpreted to constitute a surrogate marker for BT[430]. This is corroborated by the identification of bacterial species commonly found in the gut of patients with cirrhosis, in their ascitic fluid[431]. However, reports that non-gut associated microbes have been demonstrated suggests that BT is not limited to translocation from the gut[431]. A better understanding of the bacteria present in ascites and how and if they potentially lead to the development of SBP, could assist improvement of management strategies.

Techniques using bacterial DNA have identified the same bacteria grown in culture positive cases of SBP[432]. These methods therefore provide a conceivable alternative to culture based techniques and their increasingly recognised limitations[433,434]. These techniques may therefore permit tailoring of antibiotics, improving outcomes and preventing subsequent antibiotic resistance, well known to occur in patients prescribed prophylactic antibiotics for SBP[373] and in those who have had recent contact with the healthcare system[435]. Furthermore, it may augment identification of organisms in patients already receiving antibiotic therapy, particularly as more of the organisms are

likely to be Gram-positive[436,437]. In fact, bacterial DNA techniques could markedly improve management of patients with Gram-positive infections, since these are reported to have significantly lower, non-diagnostic, ascitic fluid neutrophil counts than Gram-negative infections[436].

Concentration of bacterial DNA is reported to positively correlate in patients with SBP to a cytokine response, including TNF[438,439]. This is significant since TNF levels are related to the development of renal failure in patients with SBP[440], which is associated with an increased risk of death[402]. Presence of bacterial DNA in patients with culture negative non-neutrocytic ascites is also reported to independently predict 1 year mortality after exclusion of bacteraemia, SIRS, upper gastrointestinal bleeding and prior antibiotic treatment[441]. Bacterial DNA may therefore be a useful biomarker in the management of patients with cirrhosis with or without ascites, but may also facilitate a better understanding of the pathogenesis and provide therapeutic targets and identification of other biomarkers.

1.5 Literature review summary

The major global health burden attributed to CLD is expected to escalate as increasing numbers of patients present with cirrhosis, due to poor uptake of HCV therapy, and increasing rates of alcohol abuse and obesity[275]. The development and validation of biomarkers is thus paramount to improving health outcomes of patients with CLD, as it will enable better more reliable detection, monitoring and management of subjects with CLD and factors identified to be integral to disease progression and/or poorer outcomes.

Alcohol is an important primary and comorbid cause of liver injury that is often unidentified. The efficacy of structured screening questionnaire methods has been demonstrated[33], but there is little or no data regarding their use for patients being evaluated for liver disease. Due to the low uptake of alcohol screening questionnaires[48,49], considerable work has been invested into identifying objective biomarkers of alcohol use. The most specific serum biomarker is CDT, which with the introduction of a standardised method using HPLC has increased clinical utility. However, its use in patients with liver

disease is unclear, particularly since patients with cirrhosis are reported to have di-tri bridging that can result in inaccurate quantification[132,133].

Detection and quantification of hepatic fibrosis is important to identify patients with CLD who require monitoring and to determine prognosis and make therapeutic decisions. With the increasing prevalence of CLD, especially NAFLD, it is particularly important to be able to identify subjects with advanced fibrosis who are at risk of the associated complications. The ELF test provides a non-invasive assessment of fibrosis severity and is now commercially available in Australia. However, it has not been validated in an Australian population. Furthermore, the influence of clinicopathological variables present in patients with CLD on the utility of the ELF score (using manufacturer's cut-off) is not clear.

Ascites is a significant complication of end-stage liver disease that heralds the onset of decompensation. Patients with cirrhosis and ascites have significant increase in morbidity and mortality with increased hospital admissions and health care costs[274]. The identification of clinical parameters associated with development or recurrence of complications and hospital admissions should lead to an improved approach to hospital use, better patient outcomes and cost savings. Methods using bacterial DNA provide a conceivable alternative to culture based techniques for managing patients with SBP. Bacterial DNA may also be a useful biomarker for predicting patients with poor outcomes and more hospital admissions, but may also facilitate a better understanding of the pathogenesis and provide therapeutic targets and identification of other biomarkers for use in clinical practice.

1.6 Specific thesis aims

- Ascertain the level of documentation of alcohol intake for patients seen in the hepatology clinic and the concordance between documented alcohol histories and those obtained using structured alcohol questionnaires.
- Determine the utility of the standardised CDT method in patients with CLD and the impact of clinical variables.

- Evaluate the diagnostic accuracy of the ELF test at identifying advanced fibrosis in a large cohort of Australian patients with CLD of mixed aetiology and the influence of clinicopathological variables.

- Examine use of hospital services by patients with cirrhosis and ascites requiring paracentesis and identify factors associated with early unplanned readmission.
- Investigate whether microbial burden, determined by quantification of bacterial DNA in ascitic fluid, affects clinical outcomes and the potential role of clinical variables, bacterial profile and the innate immune system.

CHAPTER 2

GENERAL METHODS

2.1 Patients and clinical data

2.1.1 Patient recruitment

All patients included in the studies were recruited from the Princess Alexandra Hospital, Brisbane, Australia. Informed written consent was obtained from each patient and the protocols were approved by the Metro South Health and The University of Queensland Human Research Ethics Committees (Appendix 1).

2.1.2 Inclusion and exclusion criteria

Specific inclusion and exclusion criteria for each study are outlined in the methods section of the relevant chapters.

2.1.3 Collection of demographic and clinical data

Demographic and clinical data were obtained from research nurse interview at the time of liver biopsy, interviews with patients, longitudinal review of the medical chart and hospital computer databases, including: the hospital based corporate information system; outpatient scheduling information management system; hospital radiology database; hospital endoscopy database; hospital pathology database; and “The Viewer”, a database documenting pathology, medications, medical imaging and hospital encounters within the public health service of Queensland. The tools and questionnaires used to collect data are in the appendices (Appendix 2-6).

2.2 Laboratory data

2.2.1 Blood sample collection and processing

Blood samples were drawn at the time of liver biopsy following an overnight fast for 8-10 hours or at time of interview, as specified in the methods section of the relevant results chapters.

2.2.1.1 Serum separation

Blood collected in serum tubes was centrifuged at 900x*g* for 10 minutes or processed with the peripheral blood mononuclear cells separation protocol (21 minutes). Serum was aliquoted into 2ml safe-lock tubes and stored at -80ºC or -20ºC until subsequent analysis.

2.2.1.2 Separation of peripheral blood mononuclear cells (PBMC)

Blood (approximately 10ml) collected into ethylene diamine tetraacetic acid (EDTA) tubes was layered on top of 4ml Ficoll-Paque™ Plus (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) in a 15ml tube and centrifuged at 900x*g* for 21 minutes (acceleration 3/deceleration 3). The PBMC layer was carefully pipetted off and placed in a 10ml tube and filled to 10ml with media (Dulbecco's modified eagle medium (Invitrogen, Mulgrave, Australia), and centrifuged at 500x*g* for 5 minutes. The supernatant was then removed and the pellet resuspended in 10ml media, which was then divided into 2 tubes containing 7.5ml and 2.5ml and centrifuged again at 500x*g* for 5 minutes. For the 7.5ml tube the supernatant was removed and the cells resuspended in 350µl of fetal bovine serum (Invitrogen, Mulgrave, Australia) in a 1.5ml cryotube and then 350µl of 20%DMSO/media added drop by drop. The cryotube was placed in a freezing apparatus (FrostyBoy) and stored at -80°C for at least 4 hours or overnight before transfer to liquid nitrogen. For the 2.5ml tube the supernatant was removed and the pellet resuspended in TRIzol (Sigma-Aldrich, Castle Hill, Australia) and stored at -80°C in 2ml safe-lock tubes.

2.2.2 Assessment of carbohydrate deficient transferrin (CDT)

The %CDT in serum was quantified as specified in the methods section of results chapters 4 and 5 by Pathology Queensland, Brisbane, Australia.

2.2.3 Determination of ELF score

The ELF score was quantified as specified in the methods section of results chapter 6 by Pathology Queensland, Brisbane, Australia. The imprecision of the ELF score components (HA, PIIINP, TIMP-1) was determined from 22 replicates at three control levels for each analyte.

2.2.4 Non-routine biochemical analyses

Serum samples stored at -20°C were used to determine: CRP using the UniCel DxC800 analyser (Beckman Coulter, Pasadena, California, USA); IgA using the IgA1 BNII kit (Invitro, Noble Park North, Victoria, Australia); PCT using the mini vidas automated immunoassay analyser (bioMérieux, Durham, North Carolina, USA); zinc using the Pentra Spectrophotmeter (HORIBA Medical, Irvine, California, USA), by Pathology Queensland, Brisbane, Australia.

2.3 Histopathological examination

Percutaneous liver biopsies were performed at the Princess Alexandra Hospital following an overnight fast for 8-10 hours. The core was originally fixed in 10% buffered formalin, embedded in paraffin, and standard 5μm sections cut and stained with haematoxylin-eosin for histopathological assessment. The liver biopsy reports were used to confirm aetiology, stage and grade of disease. Further analysis of liver biopsies was performed as specified in the methods section of the relevant results chapters by Dr Clouston (Envoi Pathology, Brisbane, Australia) or Dr Lampe (Pathology Queensland, Brisbane, Australia).

2.4 Cell isolation and DNA purification from ascites samples

Ascites fluid was collected under sterile conditions. 14ml was centrifuged at 5000x*g* for 10 minutes in sterile 15ml conical tubes (Falcon). The cell pellet was then resuspended in 200μl sterile Dulbecco's phosphate buffered saline (PBS). DNA was extracted from cell pellets under sterile conditions using the QIAamp DNA Mini kit in accordance with the manufacturer's instructions (Qiagen, Venlo, Netherlands). Extracted DNA was quantified using a Nanodrop lite spectrophotometer (Thermo Scientific, Wilmington, USA) and then stored at -20ºC.

2.5 Polymerase chain reaction (PCR)

Real time PCR was performed with a Stratagene Mx3000P thermal cycler (Agilent Technologies Inc., Santa Clara, USA) using 2x SYBR® Select Master Mix (Applied Biosystems, Mulgrave, Australia). Additional PCR assays were performed with a Mastercycler pro (Eppendorf, Hamburg, Germany) using Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA). In addition to the qPCR melt curve analysis, PCR products were assessed by agarose gel electrophoresis on a 1.5% gel containing 0.01% ethidium bromide in 0.5 x Tris-Borate-EDTA at 90 volts for approximately 45-60 minutes with the molecular marker HyperLadder 1. Bands were visualised with ultraviolet transilluminator and images captured.

2.5.1 Primer selection

Three commonly used primer pairs amplifying different regions of the bacterial 16S rRNA gene were provided by our collaborators for assessment (Table 2.1). DNA extracted from the ascites cell pellet, collected from a patient with proven SBP (culture positive (*Streptococcus mitis*), neutrophil count ≥250/mm^3), was used to assess primer specificity. PCR products were assessed by gel electrophoresis and the primer pair 517F/803R selected because it produced a single amplicon, in comparison to the other primer combinations tested, which exhibited non-specific amplification (Figure 2.1).

Table 2.1. Primer pairs assessed and their sequences

Primer	Amplicon Nucleotides (n)	Sequence 5'-3'
803F	589	GGATTAGATACCCYGGTAG
1392R		ACGGGCGGTGTGTRC
347F	456	GGAGGCAGCAGTRRGGAA
803R		CTACCRGGGTATCTAATCC
517F	286	GCCAGCAGCCGCGGTAA
803R		CTACCRGGGTATCTAATCC

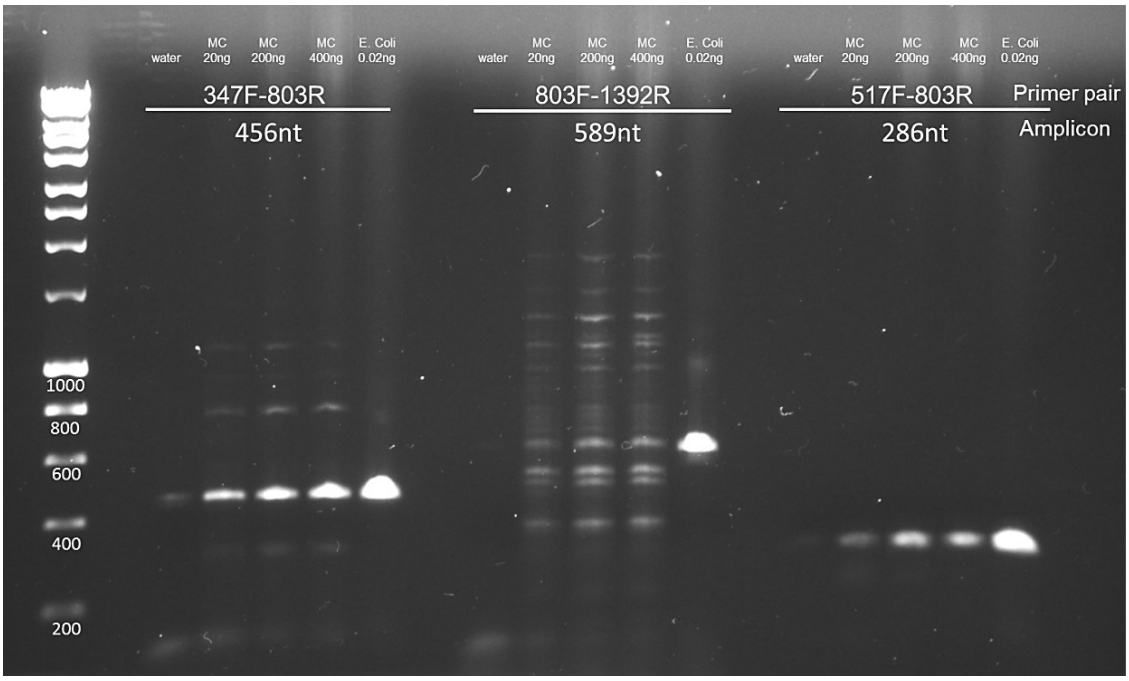


Figure 2.1. Agarose gel assessing the amplicon and presence of non-specific PCR products of 16S rRNA primer pairs using different template amounts (20ng, 200ng and 400ng) from the patient with SBP (MC) and negative (water) and positive (*E.coli* 0.02ng) controls.

2.5.2 Optimisation of primer concentration

Real time PCR was performed using a dilution series (1μM, 0.8μM, 0.4μM and 0.2μM) of the 517F/803R primers with 10ng or 200ng DNA template for the known SBP patient, 2 non-neutrocytic ascites patients and for a negative (water) and positive (E.coli) control. The optimal primer concentration, providing the optimal threshold cycle (C_t) without amplification of non-specific products, was between 0.4μM and 0.8μM, thus a concentration of 0.5μM was chosen for further experiments.

2.5.3 Determining optimal template amount

The optimal amount of template was determined by real time PCR using a dilution series (100ng, 50ng, 10ng, 5ng, 1ng, 0.5ng) of template from the known SBP patient. 50ng of template produced the strongest amplicon with the least non-specific product and primer dimer artefacts on gel electrophoresis (Figure 2.2).

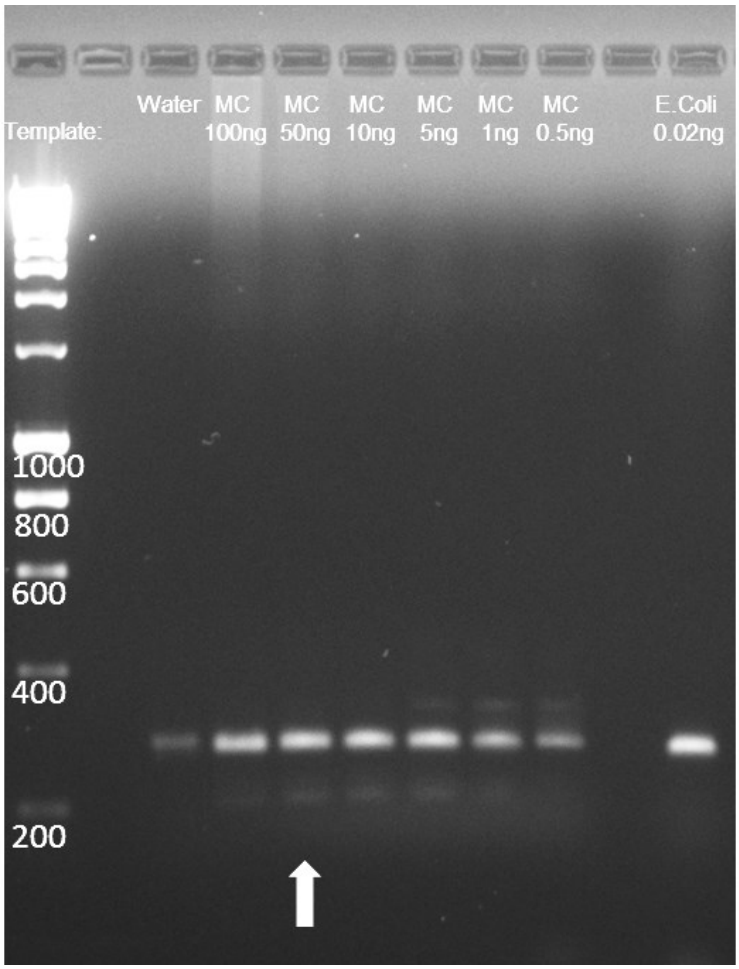


Figure 2.2. Agarose gel assessing the amplicon and presence of non-specific products and primer artefacts for the 517F/803R primers using a template dilution series with DNA from the ascites cell pellet from the patient with known SBP (MC), compared to negative (water) and positive (*E.coli*) controls.

2.5.4 Optimisation of PCR conditions

Gradient PCR was performed to determine the ideal primer annealing temperature using DNA extracted from the ascites cell pellet from the patient with known SBP. A temperature of 57ºC was selected because the strongest amplicon with the least non-specific product and primer artefacts occurred between 56.7ºC and 57.9ºC (Figure 2.3).

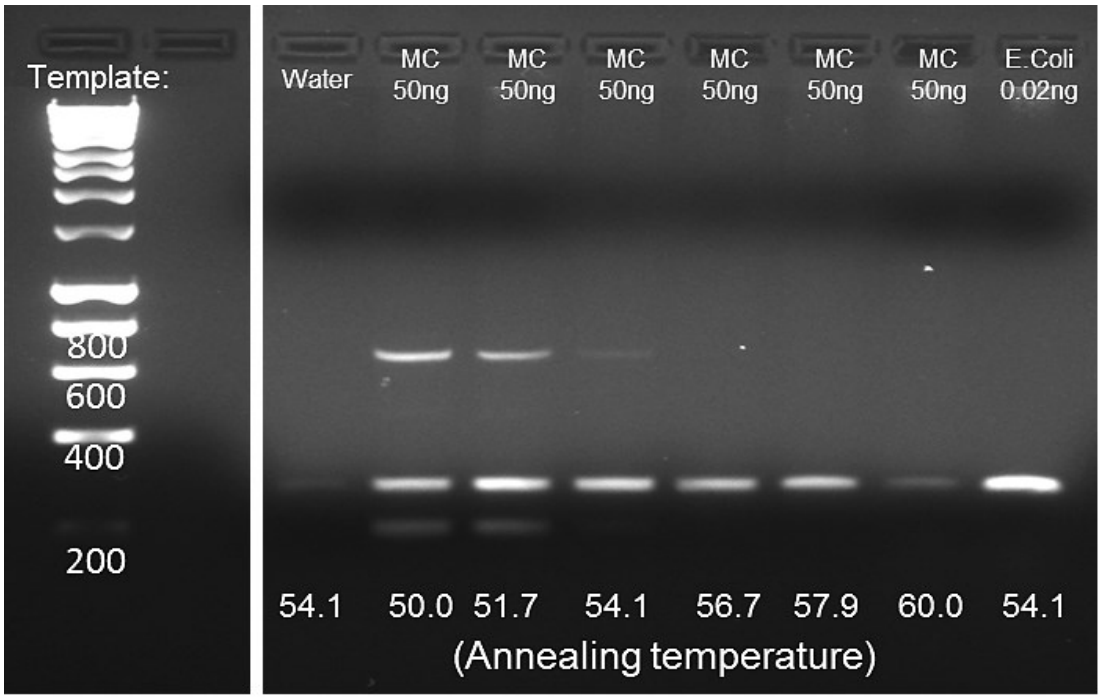
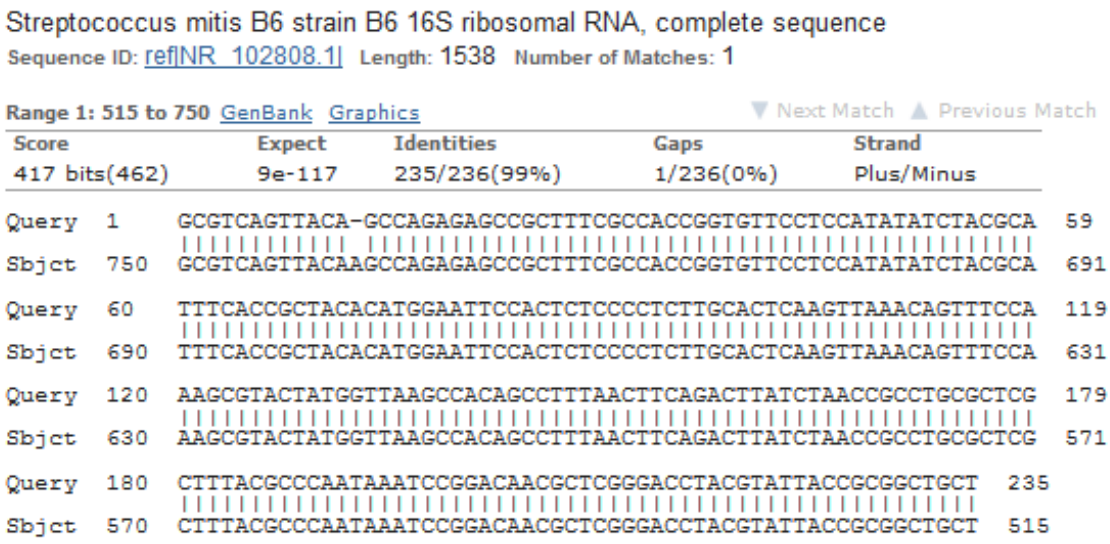


Figure 2.3. Agarose gel assessing the amplicon and presence of non-specific artefact for the 517F/803R primers for a temperature gradient from 50.0ºC to 60.0ºC, using 50ng of DNA template extracted from the ascites cell pellet from the patient with known SBP (MC), compared to negative (water) and positive (*E.coli*) controls.

2.5.5 Proof of concept

The amplicons for the patient with known SBP and for the positive control (*E.coli*) were cut out of the agarose gel and the DNA extracted and purified using the QIAquick gel extraction kit, in accordance with the manufacturer's instructions (Qiagen, Venlo, NL). DNA was made up to 1ng/µl in 12µl with 10µM of 517F or 803R primer, and sent to The Australian Genome Research Facility (AGRF, University of Queensland, Brisbane, Australia) for Sanger sequencing. The Basic Local Alignment Search Tool (BLAST) correctly identified the *E.coli* and *Streptococcus mitis*, the organism identified by microbiological techniques for the known SBP patient (Figure 2.4).



Streptococcus mitis B6 strain B6 16S ribosomal RNA, complete sequence
Sequence ID: ref|NR_102808.1| Length: 1538 Number of Matches: 1

Range 1: 515 to 750 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
417 bits(462)	9e-117	235/236(99%)	1/236(0%)	Plus/Minus

```
Query  1    GCGTCAGTTACA-GCCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATCTACGCA  59
            ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  750  GCGTCAGTTACAAGCCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATCTACGCA  691

Query  60   TTTCACCGCTACACATGGAATTCCACTCTCCCCTCTTGCACTCAAGTTAAACAGTTTCCA  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  690  TTTCACCGCTACACATGGAATTCCACTCTCCCCTCTTGCACTCAAGTTAAACAGTTTCCA  631

Query  120  AAGCGTACTATGGTTAAGCCACAGCCTTTAACTTCAGACTTATCTAACCGCCTGCGCTCG  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  630  AAGCGTACTATGGTTAAGCCACAGCCTTTAACTTCAGACTTATCTAACCGCCTGCGCTCG  571

Query  180  CTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGCTGCT  235
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  570  CTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGCTGCT  515
```

Figure 2.4. Basic Local Alignment Search Tool (BLAST) alignment of 16S rRNA amplicon sequence (803R, "Query") from the patient with *Streptococcus mitis* cultured SBP, confirming the identification of *Streptococcus mitis* (NR_102808.1, "Sbjct"), with high confidence (Expectation value 2 x 10^{-119}).

2.6 Assessment of bacterial density and bacterial community analyses

Microbial 16S rRNA DNA present in the ascites cell pellet was quantified by real-time PCR with reference to a standard curve generated from purified *E.coli* DNA as described in results chapter 8. All 25 patients had 16S pyrosequencing performed by mrdna.com (Molecular Research (MRDNA), Shallowater, Texas, USA) on the Illumina HiSeq platform. Bacterial community analysis was performed by Dr Rogers (South Australian Medical Research Institute, Adelaide, Australia) as specified in the methods section of results chapter 8.

2.7 Ascites cell isolation and flow cytometric analysis

Ascites cells were isolated as described in the methods section of results chapter 8. Flow cytometric analysis was performed and analysed by Dr Irvine, Dr Arthur and Dr Melino (Centre for Liver Disease Research, Brisbane, Australia) as outlined in the methods section of results chapter 8.

2.8 General statistical analysis

Statistical analysis was performed as described in the methods section of the results chapters. Statistical advice and support was provided by Dr Ballard and Professor O'Rourke (Statistics unit, QIMR Berghofer Medical Research Institute, Brisbane, Australia).

CHAPTER 3

ASSESSMENT OF ALCOHOL HISTORIES OBTAINED FROM PATIENTS WITH LIVER DISEASE: OPPORTUNITIES TO IMPROVE EARLY INTERVENTION

Fagan KJ[1,2], Irvine KM[2], Kumar S[2], Bates A[2], Horsfall LU[1,2], Feeney GF[3], Powell EE[1,2]. Assessment of alcohol histories obtained from patients with liver disease: opportunities to improve early intervention. *Intern Med J*. 2013; **43**: 1096-102.

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Contributor	Statement of contribution
Kevin Fagan *DM candidate*	Conceived and designed study (70%) Collected data (70%) Data analysis (80%) Wrote the paper (100%) Edited the paper (20%)
Katharine Irvine *Research fellow*	Data analysis (10%) Edited the paper (20%)
Santosh Kumar *Medical student*	Collected data (10%)
Anthony Bates *Medical student*	Collected data (10%)
Leigh Horsfall *Clinical research coordinator*	Collected data (10%)
Gerald Feeney *Addiction medicine specialist*	Provided guidance with design of study and interpretation of results Edited paper (20%)
Elizabeth Powell *Principal supervisor*	Conceived and designed study (30%) Data analysis (10%) Edited the paper (40%)

Abstract

Background: Alcohol is an important primary and co-morbid cause of liver injury in patients referred for investigation and management of liver disease. Early assessment and documentation of alcohol consumption is therefore essential, and recommended in both general practice and hospital settings.

Aims: To determine the extent and accuracy of documentation of alcohol consumption in patients referred for evaluation of liver disease.

Methods: Patients were interviewed using a structured questionnaire. The medical records of all patients interviewed were reviewed to obtain information from the referral letter and the hepatology consultations.

Results: 83 patients were surveyed. Only 14 referrals had an informative alcohol history, despite 27 patients admitting risky alcohol consumption at the initial hepatology consultation. 90% of initial consultations had an informative alcohol history documented, whereas only 56% of patients attending a follow-up appointment had informative documentation. Assessment of alcohol consumption was comparable between the hepatology consultation and the structured questionnaire, but 4 subjects had substantially different alcohol histories. AUDIT identified all patients reporting harmful alcohol consumption on the questionnaire.

Conclusions: Hazardous alcohol use is prevalent in subjects attending hepatology clinics, but informative alcohol histories which are crucial to patient management, are rarely documented in referrals. Screening tools improve documentation and accuracy of alcohol histories and their use by general practitioners and hospital clinicians would improve detection rates of hazardous drinking and allow earlier intervention. Systematic use of screening tools in hepatology clinics will provide opportunities for education and reinforce recommendations to reduce hazardous or harmful alcohol consumption.

3.1 Introduction

Alcohol consumption remains an important primary cause of liver injury in Australia[442] and is a common comorbid factor accelerating the progression of other chronic liver diseases (CLD)[443]. The “safe” limit of alcohol intake in those with CLD remains unclear[15], but the synergistic effects of alcohol have been demonstrated to occur at lower levels[13,14] than the 40-80g/day identified as an independent environmental risk factor[444]. Assessment and documentation of alcohol consumption is therefore an essential component of hepatology consultations, as this information significantly impacts the patient’s current and future care.

Despite policies and regulations to reduce alcohol-related harm[445,446], alcohol misuse remains a major health and social problem in Australia[447]. General practitioners (GPs) have a key role as advocates for health promotion and are in a strong position to identify hazardous and harmful alcohol consumption. In a recent report summarising GP activity in Australia, one-quarter of sampled adults reported drinking alcohol at *at-risk* levels[448]. Evidence suggests that screening for risk levels of alcohol consumption and brief intervention can be helpful and cost effective[36,449-451] and thus is recommended in both general practice and hospital settings.

Established methods for detecting risky alcohol consumption include a quantitative alcohol history and the use of screening questionnaires. Guidelines for the treatment of alcohol problems prepared for the Australian Government Department of Health and Ageing (2009) recommend the use of a quantitative alcohol history (quantity-frequency estimates) to detect levels of consumption in excess of National Health and Medical Research Council (NHMRC) guidelines in the general population[16]. This history comprises: the daily average consumption (grams/standard drinks per day) of alcohol and the number of drinking days per week (or month)[16]. Of the available screening questionnaires, the Alcohol Use Disorders Identification Test (AUDIT) is recommended by the NHMRC for use in the general population to identify current hazardous alcohol consumption[28]. It consists of 10 questions that cover the domains of hazardous

drinking, dependence symptoms and harmful alcohol use, and has been validated for use across different cultural groups.

Australian investigators have had a substantial role in the WHO Collaborative Project on Identification and Management of Alcohol-related Problems in Primary Health Care[452,453]. However in clinical practice, failure to recognise or document excessive alcohol consumption remains common, with studies suggesting that only 30% of subjects with hazardous or harmful patterns of alcohol use are identified by GPs or hospital doctors[16-21]. This may hinder the prevention and earlier management of alcohol-related liver disease (ALD), as well as the management of other CLD where alcohol is a contributing or exacerbating factor[443]. To our knowledge, there is no published data on the extent to which alcohol is underestimated as a risk factor in patients referred for evaluation of liver disease.

Therefore the aim of this study was to ascertain the level of documentation of alcohol intake in referral letters and medical records of patients seen in a hepatology outpatient clinic at a major tertiary referral hospital. Additionally, concordance between documented alcohol histories and alcohol assessment obtained by patient interview using validated alcohol questionnaires was evaluated.

3.2 Methods

The study was conducted in the hepatology outpatient clinics at the Princess Alexandra Hospital, Brisbane, during 2012. Informed consent was obtained from each patient and the protocol was approved by Metro-South-Health and the University of Queensland Human Research Ethics Committees. Hepatology consultants were informed about the research project at an intradepartmental meeting in 2011. Patients were approached and invited to participate in the study, following their hepatology consultation. Those who agreed to participate were interviewed by the research co-ordinator using a structured questionnaire and a standard drink guide[7]. The questionnaire incorporated an alcohol calendar to record alcohol consumption over the prior 4 week period and further

direct questions to determine whether the calendar reflected their usual alcohol consumption. It also included questions about the age at which regular alcohol drinking commenced, and whether they had ever had previous periods of heavy alcohol consumption, defined as ≥350g/week for females and ≥420g/week for males for ≥6 months. These questions were followed by the AUDIT and the Brief Michigan Alcoholism Screening Test (bMAST), a 10 item test derived from the 25 item Michigan Alcoholism Screening Test that is widely used in the assessment of alcohol dependence[47].

83 patients consented to participate, 28 of whom had attended their initial consultation and 55 a follow-up consultation. All patients' medical records were reviewed to obtain information from the referral letter and the hepatology consultation on the day of interview. For those attending a follow-up appointment on that day, information from their initial hepatology consultation was also retrospectively obtained. An informative alcohol history was defined as one that enabled the subject to be confidently assigned to an alcohol group based on their current alcohol consumption: Group 0, no alcohol consumption; Group 1, less than recommended maximum weekly allowance (females >0-140g/week, males >0-210g/week); Group 2, greater than recommended maximum weekly allowance (>RWA) but less than heavy (females >140-<350g/week, males >210-<420g/week); Group 3, heavy (females ≥350g/week, males ≥420g/week). Alcohol groups were devised to separate the cohort into those consuming no alcohol, those drinking at a level unlikely to cause liver injury and those drinking at a level that may cause liver injury (based on epidemiological data)[9-12]. The latter group was further divided to distinguish "heavy" drinkers.

Diagnosis of liver disease was based on standard biochemical and serological assays and histological assessment of the liver biopsy (if performed), as previously described[454].

The degree of agreement between alcohol group classification by questionnaire and consultation was calculated using the Kappa score (GraphPad, Software Inc. La Jolla, California, USA).

3.3 Results

3.3.1 Alcohol history provided in referral

Of the 83 patients surveyed, 42 were referred by a GP, 28 were referred by another hospital specialist or peripheral hospital, 2 were followed up after a hospital admission under the care of hepatology, and 11 referrals were not available for review. The mean age of the referred patients was 49.8 ± 12.2 years and 71.1% were male. Of the 72 patients with a reviewed referral, the reason for referral was management of hepatitis C virus (HCV) infection in 33 patients (45.8%), cirrhosis or its complications including hepatocellular cancer in 12 (16.7%), chronic hepatitis B virus (HBV) in 7 (9.7%), abnormal liver enzymes in 7 (9.7%), elevated serum ferritin in 4 (5.6%), ALD in 4 (5.6%), immune-mediated liver disease in 3 (4.2%) and follow-up after a hepatology hospital admission in 2 (2.8%).

24 (34.3%) of the reviewed referrals included a comment regarding alcohol history, although only 14 of these were informative (Figure 3.1). Of these, 5 were reported to be drinking hazardous or harmful levels of alcohol.

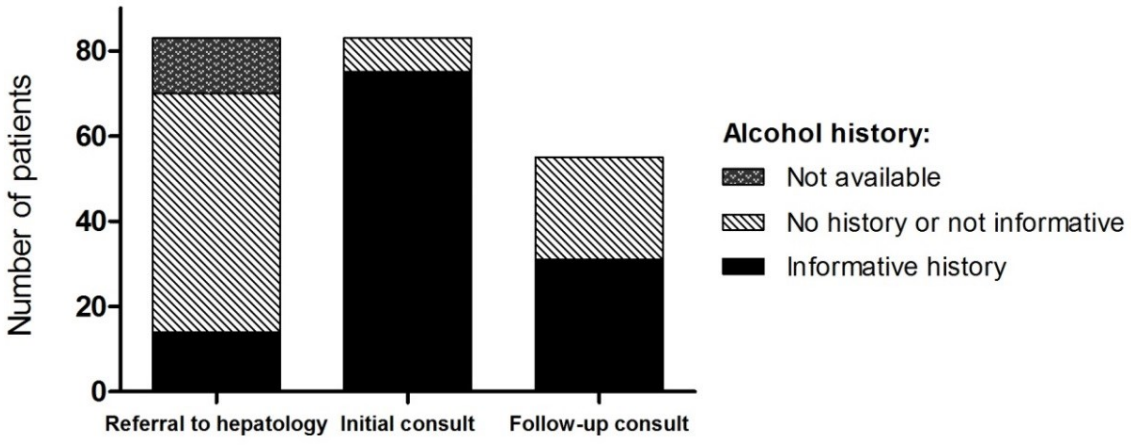

Figure 3.1. Number of patients for whom an informative alcohol history was documented in the referral and hepatology consultations.

3.3.2 Alcohol history provided at initial hepatology consultation

At the initial outpatient hepatology consultation, 54 patients were seen by a consultant hepatologist and 29 by an advanced trainee or fellow training in gastroenterology and hepatology. In contrast to the referral letters, 90.4% (75) patients had an informative alcohol history documented in the medical record during their initial consultation. Of the 8 patients with no documentation, 3 referrals were unavailable, 4 referrals did not provide an alcohol history and 1 referral stated that the patient was a “current drinker” but did not quantify the alcohol consumption. The overall mean waiting time from referral to first consultation was 1.4 years (median 0.5 years).

The median reported alcohol consumption at the time of initial hepatology consultation was 71 (0-1558) g/week. Subjects were classified into the previously defined alcohol groups: Group 0: 22 (29.3%); Group 1: 26 (34.7%); Group 2: 11 (14.7%); Group 3: 16 (21.3%). 8 patients had no documented alcohol history at their initial consultation, all of whom were seen by a consultant. The lack of alcohol documentation in referral letters was not confined to non-drinkers; only 4 of 27 patients in high-risk alcohol groups (2 or 3) were referred with an informative alcohol history, and for one the referral could not be found (Figure 3.2). Interestingly, 3 of the 5 patients identified in their referral as consuming alcohol at hazardous or harmful levels had substantially reduced or ceased alcohol intake at the time of initial hepatology consultation. (Figure 3.2)

3.3.3 Alcohol history provided at follow-up hepatology consultation

55 patients were surveyed after a follow-up appointment with the hepatology outpatient department. These patients had been attending the department for a median of 1.2 years (0.1-16.3 years). At this hepatology consultation, 33 patients were seen by a consultant hepatologist, 21 by an advanced trainee or fellow training in gastroenterology and hepatology and 1 patient was reviewed by a basic trainee. In contrast to the initial consultation, only 31 (56.4%) patients had an informative comment about current alcohol intake documented in the medical record during the review consultation (Figure 3.1).

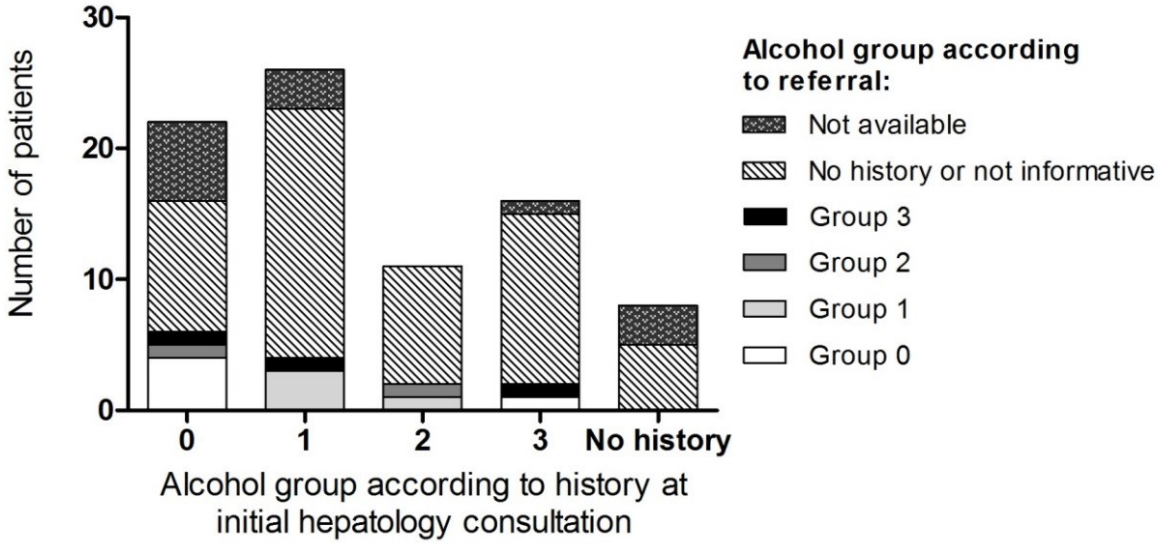

Figure 3.2. Comparison between the numbers of patients within each alcohol group according to alcohol histories documented in referrals and in the initial hepatology consultation.

The median reported alcohol consumption at the time of the hepatology review consultation was 20 (0-2100) g/week. The number of subjects per alcohol group was: Group 0: 14 (25.5%); Group 1: 11 (20%); Group 2: 3 (5.5%); Group 3: 3 (5.5%). Of the 24 patients *without* an informative comment about current alcohol consumption, 2 had been documented to be consuming harmful levels of alcohol at the initial consultation, 14 were cirrhotic and 11 had a prior history of heavy alcohol intake.

3.3.4 Information obtained from alcohol questionnaires

Of the 83 patients surveyed, 28 completed the alcohol questionnaire after their initial hepatology consultation and 55 after a follow-up consultation. The results of the survey are detailed in Table 3.1. Overall, 36 (43.4%) patients were not drinking, 25 (30.1%) patients were classified as Group 1, 6 (7.2%) patients as Group 2 and 16 (19.3%) patients as Group 3. 63 (75.9%) reported a history of previous heavy alcohol consumption.

Table 3.1. Demographic and clinical information for patients completing the alcohol questionnaire.

	Alcohol questionnaire completed following:	
	Initial hepatology consult	**Follow-up hepatology consult**
Number of patients (n, %)	28 (33.7%)	55 (66.3%)
Gender (n, % male)	22 (78.6%)	37 (67.3%)
Age (yrs) (mean ± SD)	49.2 (±13.3)	50.1 (±11.8)
Ethnicity (n, % Caucasian)	22 (78.6%)	49 (89.1%)
Disease aetiology		
HCV (n, %)	15 (53.6%)	21 (38.2%)
HBV (n, %)	3 (10.7%)	5 (9.1%)
ALD (n, %)	7 (25.0%)	17 (30.9%)
NAFLD (n, %)	0 (0.0%)	4 (7.3%)
Autoimmune (n, %)	2 (7.1%)	4 (7.3%)
Other† (n, %)	1 (3.6%)	4 (7.3%)
Cirrhosis (n, %)	16 (57.1%)	29 (50.9%)
Years attending hepatology clinic (median ± range)	N/A	1.2 (0.1-16.3)
Ever heavy alcohol consumption (n, %)	24 (85.7%)	39 (70.9%)
Alcohol groups‡		
Group 0 (n)	8	28
Group 1 (n)	6	19
Group 2 (n)	2	4
Group 3 (n)	12	4

Table 3.1 notes. †Other: haemochromatosis (n=2), cryptogenic liver disease (n=2), HBV/HCV co-infection (n=1). ‡Subjects were grouped according to alcohol consumption: Group 0: 0 g/wk; Group 1: females >0-140 g/wk; males >0-210 g/wk; Group 2: females >140-<350 g/wk, males >210-<420 g/wk; Group 3: females ≥ 350 g/wk, males ≥ 420 g/wk.

58 subjects had an alcohol history recorded in the medical record on the day of the alcohol questionnaire. Documentation of alcohol consumption during the hepatology consultation and by the targeted questionnaire were comparable, with the majority of subjects being assigned to the same alcohol group in both cases (Figure 3.3, kappa score=0.67, Standard Error=0.08, weighted kappa=0.79). Despite this concordance, 4 subjects had substantially different alcohol histories recorded in the medical record; 3 were classified as alcohol groups 0 or 1, and 1 as group 2, at a time when the alcohol questionnaire elicited hazardous or harmful alcohol consumption. A further 5 subjects were assigned to a higher alcohol group according to the history gained from the alcohol questionnaire. This is important since 4 of these 5 had a history of prior heavy alcohol consumption and the other subject was undergoing treatment for hepatitis C.

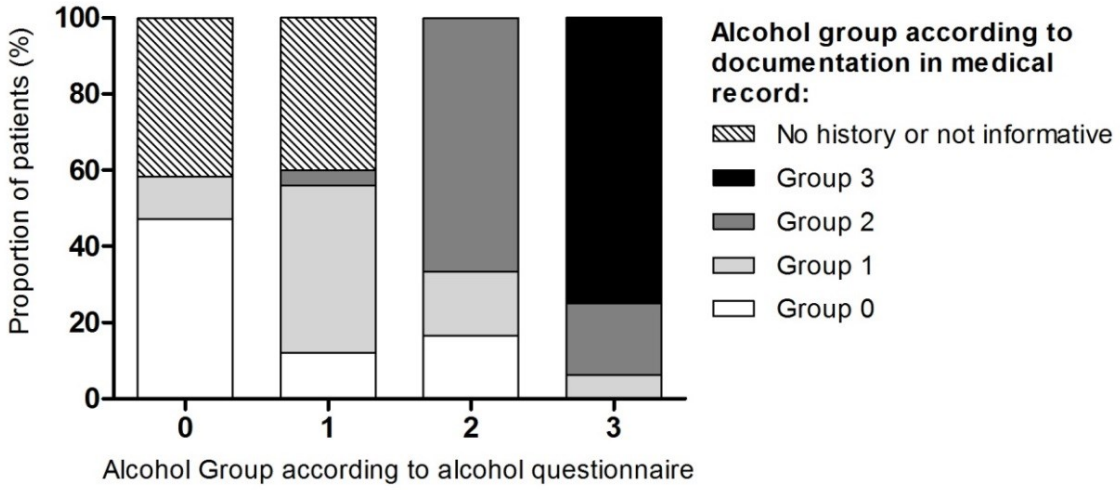

Figure 3.3. Concordance of alcohol groups according to the alcohol histories documented at the time of interview and obtained using the structured questionnaire.

All patients drinking >RWA or “heavily” on the basis of the alcohol questionnaire had an AUDIT score >8, indicative of hazardous or harmful alcohol use over the past year, as well as possible alcohol dependence. 17 patients who were abstinent or drinking <RWA also had an AUDIT score >8 (Figure 3.4), however all but 2 of these had a history of heavy alcohol intake which they had recently ceased. The other 2 patients had reportedly reduced their heavy alcohol intake more than one year prior to the assessment. For the 63 who admitted a prior history of heavy alcohol consumption in the interview, AUDIT indicated alcohol dependence in only 15 men (AUDIT>15) and 9 women (AUDIT>13). However, the bMAST score was >6, indicating a high probability of alcohol dependence in 22 of these 24, and in another 11 men and 3 women that the AUDIT did not identify.

Of the 27 patients classified as Group 2 or 3 at the initial hepatology consultation, 13 completed the alcohol survey on the same day as their follow-up appointment. Of these subjects seen at follow-up, 9 (69.2%) were now not drinking or classified as Group 1.

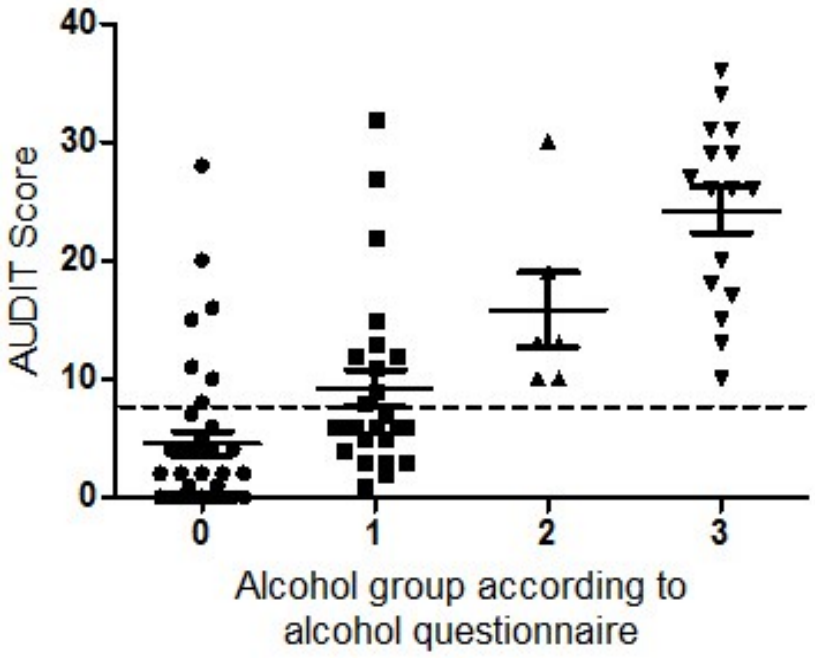

Figure 3.4. AUDIT scores within each alcohol group.

3.4 Discussion

Alcohol is an important primary and co-morbid cause of liver injury in patients referred for investigation and management of liver disease. Documentation of a quantitative alcohol history is clearly important in the liver clinic for diagnostic purposes and to assist clinical decisions and treatment plans. This study was undertaken to determine the extent and accuracy of documentation of alcohol intake in patients referred for evaluation of liver disease. The data show that only a third of referrals from general practitioners or other specialty units provided any comment regarding alcohol intake, and less than 20% provided a quantitative alcohol history. In contrast, the majority of patients (90%) had an alcohol history documented during the initial hepatology consultation. These findings are consistent with earlier studies suggesting that less than one-quarter of patients seen in general practice[455] and less than 50% of hospital in-patients[20] are routinely asked about alcohol use. In the latter study, 26% of problem drinkers (defined as an AUDIT score ≥8) had no alcohol history in the doctor's record[20].

The lack of referral documentation of alcohol intake is concerning, because at initial hepatology assessment, at least one-third of patients were drinking alcohol at hazardous or harmful levels. These patients had been waiting a mean of 1.4 years for their initial hepatology consultation and earlier intervention by the referring clinician to reduce hazardous alcohol consumption, particularly in patients with suspected liver disease, is imperative. Indeed, 71% of those referred with "abnormal liver enzymes" and 50% of those referred with "raised ferritin" were diagnosed with ALD. It is not clear whether referring clinicians were aware of the patients' alcohol history, since clinical information provided in the referral letter is often incomplete[456] and medical records may under-report delivery of some patient services, particularly details of lifestyle counselling[457]. Nevertheless, despite the limitations of the data collected, it is clear that excessive alcohol consumption needs to be detected and managed appropriately in the community, while patients are awaiting specialist outpatient review. Our data support a recent examination of general practice activity in Australia[448] suggesting that an alcohol intervention is infrequently delivered

within the GP encounter. Provision of counselling/advice regarding alcohol was recorded in only 0.4% of all encounters[448] and did not differ from the recorded rate of alcohol counselling a decade earlier[458].

Somewhat surprisingly, informative alcohol histories were recorded in only around 50% of follow-up hepatology consultations. This lack of documentation is concerning, since 71% of the patients seen at follow-up had a prior history of heavy alcohol consumption and recidivism is a significant risk at any time following abstinence. In addition, it is recognised that many of the drinking public do not know or understand alcohol guidelines[459] and thus recurrent patient visits provide multiple opportunities to educate and reinforce alcohol recommendations over time. Repeated assessment of alcohol consumption is important irrespective of the primary liver disease, as alcohol may be one of a number of factors causing liver injury. Of the 27 patients drinking at harmful levels when initially assessed, >50% had chronic hepatitis C infection, and in these patients rate of fibrosis development is exacerbated by daily alcohol consumption[460].

Since biomarkers for alcohol abuse lack sensitivity in patients with CLD[454], clinical history and self-report screening tests are considered to be the most efficient way to identify at-risk patients[461]. Allowing for missing data, agreement between documented alcohol history and alcohol questionnaire was generally good. However, in comparison with the alcohol history documented in the medical record, the screening questionnaires identified 4 patients with a substantially higher alcohol intake. A further 5 subjects were classified to a higher alcohol group by the alcohol questionnaire, which had clinical implications for all of them. The AUDIT component of the questionnaire identified all “at risk” drinkers, including those who had recently cut down. Combination with a short alcohol calendar was helpful to quantify current alcohol consumption as well as determine the pattern of drinking and type of alcohol consumed. As expected in comparison to AUDIT, bMAST was less effective in detecting people with hazardous alcohol intake, but performed better at identifying patients most likely to be alcohol dependent. Thus although

bMAST is not advocated as a screening tool in primary practice,[16] it was helpful in our hepatology cohort where risk of recidivism is important.

The public health benefits of national screening programs for hazardous alcohol consumption remain controversial[462]. Currently screening is recommended in settings where the prevalence of risky drinkers is likely to be high and where detection will have the most important benefit[16]. Hepatology clinics represent just such a setting, but evidence for the effectiveness of screening and brief interventions in hepatology clinics is non-existent. Barriers to alcohol screening include time constraints and a perceived fear of distancing patients[37]. However, interestingly most patients reportedly expect to be asked about alcohol consumption and link it with a higher quality of care[52]. Another important barrier is the ambivalence of physicians towards screening, which likely reflects physician education and confidence[18]. In our study, 69.2% of patients who reported drinking greater than recommended levels at their initial hepatology consultation were abstinent or drinking at low levels at a review consultation. This suggests that patients were positively influenced during or following the hepatology consultation to reduce their level of alcohol consumption. Assessment of the intervention delivered during the consultation or whether the patient was referred for more intensive treatment was beyond the scope of this study.

In summary, hazardous alcohol use is prevalent in subjects referred to hepatology clinics and thus screening by general practitioners and hospital clinicians *prior* to referral will improve detection rates and allow earlier intervention. Systematic screening of alcohol use with validated alcohol surveys of patients attending hepatology clinics will improve documentation and quantitation of alcohol consumption, and will provide opportunities for education and reinforcement of alcohol recommendations. Screening at follow-up visits may also remind physicians to initiate or reinforce brief interventions, since this leads to a reduction in the number of patients consuming harmful amounts of alcohol.

CHAPTER 4

BMI BUT NOT STAGE OR AETIOLOGY OF NON-ALCOHOLIC LIVER DISEASE AFFECTS THE DIAGNOSTIC UTILITY OF CARBOHYDRATE DEFICIENT TRANSFERRIN

Fagan KJ[1,2], Irvine KM[2], McWhinney BC[4], Fletcher LM[1,3], Horsfall LU[1,2], Johnson LA[4], Clouston AD[2], Jonsson JR[2], O'Rourke P[5], Martin J[3,6], Pretorius C[4], Ungerer JP[4], Powell EE[1,2]. BMI but not stage or etiology of nonalcoholic liver disease affects the diagnostic utility of carbohydrate-deficient transferrin. *Alcohol Clin Exp Res*. 2013; **37**: 1771-8.

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Contributor	Statement of contribution
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Katharine Irvine *Research fellow*	Data analysis (15%) Edited the paper (15%)
Brett McWhinney *Scientist*	Performed CDT analysis (45%)
Linda Fletcher *Scientist*	Collected samples (50%) Edited the paper (5%)
Leigh Horsfall *Clinical research coordinator*	Collected data (35%) Collected samples (50%)
Lambro Johnson *Scientist*	Performed CDT analysis (45%)
Andrew Clouston *Histopathologist*	Histopathological assessment (100%)
Julie Jonsson *Scientist*	Conceived and designed study (5%)
Peter O'Rourke *Biostatistician*	Data analysis (20%) Edited the paper (5%)
Jennifer Martin *Medical physician*	Guidance regarding volume of distribution Edited the paper (5%)
Carel Pretorius *Pathologist*	Performed CDT analysis (5%) Edited the paper (5%)
Jacobus Ungerer *Pathologist*	Performed CDT analysis (5%) Edited the paper (5%)
Elizabeth Powell *Principal supervisor*	Conceived and designed study (25%) Data analysis (10%) Edited the paper (40%)

Abstract

Background: A reliable biomarker is required in hepatology clinics for detection and follow-up of heavy alcohol consumption. Carbohydrate-deficient transferrin (CDT) increases with sustained heavy alcohol consumption and is the most specific biomarker of ethanol consumption. Recent introduction of a standardised method for measuring CDT has improved its clinical application. This study was designed to determine whether alcohol-independent factors influence CDT levels in patients with chronic liver disease.

Methods: The relationship between serum %CDT and self-reported history of alcohol consumption was examined in 254 patients referred for evaluation of liver disease. CDT analysis was performed on serum collected at time of liver biopsy.

Results: CDT levels were not affected by severity or aetiology of non-alcoholic liver disease. 13 of 254 subjects had a %CDT >1.7, predictive of heavy alcohol intake, 6 of whom did not acknowledge heavy drinking. 12 of these 13 subjects were suspected heavy drinkers on review of their medical records and clinical results. Conversely, not all acknowledged heavy drinkers had %CDT >1.7. Heavy drinkers with a BMI in the overweight or obese range had significantly lower %CDT than lean heavy drinkers. This persisted even when lean body weight was used as an approximation of the ethanol volume of distribution.

Conclusion: An elevated BMI reduces the diagnostic utility of CDT at higher alcohol intake in subjects with chronic liver disease using the standardized method. In a hepatology outpatient setting, this assay is likely to be useful to confirm suspicion of heavy drinking in subjects who are not overweight, but cannot reliably identify moderate drinkers or heavy drinkers who are overweight.

4.1 Introduction

Alcohol remains a major global cause of liver injury and is a common co-factor in subjects with other chronic liver diseases such as viral hepatitis, hemochromatosis and non-alcoholic fatty liver disease (NAFLD). Although the relationship between alcohol and liver injury is not entirely dose-dependent, the threshold for increased risk of cirrhosis occurs with a total lifetime intake of >100kg or a daily intake of >30 g/day[9]. In men, ingestion of >60-80 g alcohol/day for ≥10 years increases the risk of developing cirrhosis. In women, lower doses (>20 g/day) and shorter duration of alcohol consumption may produce severe liver injury[9,463]. In the presence of another chronic liver disease (CLD), the toxic threshold for alcohol remains unclear and is likely to be lower (reviewed in[15]).

Failure to recognize or acknowledge excessive alcohol consumption is common, and may interfere with the prevention and management of alcohol-related liver disease. Earlier studies estimated that only 30% of alcohol-related problems are identified by GPs and hospital doctors[18,19,464-466], although the extent to which alcohol misuse is overlooked in patients with chronic liver disease is less clear. There is a manifest need for a reliable biomarker that can be used in hepatology clinics for detection and follow-up of heavy alcohol consumption.

Various routine laboratory tests including γ-glutamyltransferase (γ-GT) and mean corpuscular erythrocyte volume (MCV) are often used as an adjunct to support a clinical suspicion of heavy alcohol intake. However, most of these tests lack sensitivity and specificity, particularly in patients with chronic liver disease. The most specific biomarker of heavy alcohol consumption is carbohydrate deficient transferrin (CDT)[119]. CDT refers to a temporary alteration in the glycosylation pattern of transferrin resulting in an increase in the relative amounts of disialo- and asialotransferrin (and a decrease in tetrasialotransferrin) that occurs as a result of sustained, moderate to high alcohol consumption (thresholds range from 50-80 grams of alcohol per day for at least 2 weeks). The altered transferrin glycosylation pattern returns to baseline levels within 2-5 weeks when alcohol is discontinued[117].

Until recently, CDT was measured by several techniques that analysed different combinations of transferrin glycoforms and reported the results in absolute *or* relative concentrations[118]. This led to uncertainty about the reliability of the test as a clinical tool to confirm alcohol abuse. However a standardised method of measuring CDT has recently been developed, using high performance liquid chromatography (HPLC) and quantification of disialotransferrin as a percentage of the total transferrin (%CDT)[119]. HPLC provides a graphic visualization of the individual transferrin glycoforms permitting the detection of factors that interfere with the analysis. Visible assessment of glycoform patterns may be important in patients with liver disease since cirrhosis has been reported to lead to poor chromatographic resolution of disialotransferrin from trisialotransferrin (di-tri bridging), a pattern that could result in inaccurate quantification[132,133]. Prior to the introduction of the standardised method, it was reported that CDT was elevated in patients with liver disease in the absence of documented alcohol abuse[129]. The accuracy of the standardised method of CDT measurement in subjects with liver disease has not been tested.

The blood alcohol concentration obtained upon consumption of a specific amount of alcohol is determined by a) the extent of “first-pass” metabolism in the liver and gastric mucosa, which is affected by a range of genetic and environmental factors, including gender, ethnicity and diet (including alcohol)[58], and b) the subject’s weight and gender, which determines the volume of distribution of ethanol. With respect to CDT testing by HPLC, an earlier study identified that heavy drinkers with a *normal* body mass index (BMI) (20-25 kg/m^2) showed higher %CDT values[118]. However the authors concluded that adjustment of reference intervals for %CDT in relation to BMI is not required, as the differences were minor and “possibly clinically non-relevant”[118].

The aim of this study was to determine in patients with chronic liver disease, whether the level of %CDT as determined by the recently standardised HPLC method, was influenced by clinical variables including BMI, aetiology or severity of liver disease.

4.2 Materials and Methods

4.2.1 Patients and clinical data

We examined the relationship between serum %CDT and self-reported history of alcohol consumption in patients referred for evaluation of liver disease. The study included 254 subjects who had undergone a liver biopsy after referral to hepatology outpatients at the Princess Alexandra Hospital, Brisbane, Australia. Informed consent was obtained from each patient and the protocol was approved by the Princess Alexandra Hospital and the University of Queensland Human Research Ethics Committees. Information regarding average alcohol intake during the preceding 2 weeks and previous average weekly consumption, was obtained from the research nurse interview at the time of liver biopsy. This was corroborated by a longitudinal review of the alcohol history documented in the medical record. Information retrieved from the medical records included basic demographic details, previously diagnosed liver disease and other medical conditions, medications and history of tobacco and recreational drug use.

Measurements of weight and height were obtained from patients at the time of liver biopsy. BMI was calculated as weight in kg/(height in meters)^2. BMI was classified as lean (<25 kg/m^2 in Caucasians, <23 kg/m^2 in Asians), overweight (25-29.9 kg/m^2 in Caucasians, 23.0 to 24.9 kg/m^2 in Asians) or obese (≥30 kg/m^2 in Caucasians, ≥ 25.0kg/m^2 in Asians). The volume of distribution (Vd) of ethanol was estimated from lean body weight using the Janmahasatian equation as this has been validated in an obese population[467]. Further, as fat has little water, lean body weight approximates Vd for ethanol.

Diagnosis of liver disease was based on standard biochemical and serological assays and histological assessment of the liver biopsy. All patients with chronic viral hepatitis had detection of circulating HCV RNA or HBV DNA by polymerase chain reaction using the Abbott m2000 RealTime System (Abbott Laboratories, Illinois, USA). No patients were receiving antiviral therapy at the time of liver biopsy and serum collection. The diagnosis of NAFLD was established by a liver biopsy specimen showing steatosis with or without

features of steatohepatitis (inflammation and hepatocyte ballooning, with or without Mallory's hyaline or fibrosis) in the setting of increased BMI and alcohol intake <20 g/day. The diagnosis of alcohol-related liver disease (ALD) was established by a history of excessive habitual alcohol intake together with physical signs and laboratory evidence of liver disease. Patients with NAFLD and ALD were negative for hepatitis B surface antigen and antibodies to HCV and HIV.

4.2.2 CDT analysis

Serum was collected at the time of liver biopsy following an overnight fast for 8-10 hours and stored at -80ºC, a condition under which the transferrin glycoform pattern is stable for very long periods[468].

CDT analysis was performed on a Waters HPLC System (Waters Corporation Milford MA USA), consisting of a 2695 Separation Module, a 2487 Dual Wavelength Absorbance Detector, a Reagent Manager Auxiliary Pump, and a Switching Valve using a Commercial Assay Kit (Clin-Rep® CDT in serum – online, Recipe, Munich, Germany) based on the IFCC-CDT-WG reference method[131,469]. A 100μl serum sample was diluted into 1 mL of an iron saturating solution, incubated in the dark at 4°C for 30 min, centrifuged at 10,000g for 5 minutes and 500μl of supernatant injected into the HPLC system. The assay employed anion exchange chromatography with a salt gradient elution to separate the CDT isoforms, and quantification by selective absorbance of the iron-transferrin complex at 460nm. After column switching, the online pre-filter was regenerated by a wash solution which removes serum matrix components to waste, prior to the next injection. CDT was defined as the percentage of disialotransferrin to total transferrin (the sum of di-, tri-, tetra- and penta- sialotransferrins), calculated as peak areas using valley to valley integration according to IFCC recommendations[131]. Accuracy and imprecision of the CDT analysis method was checked in each run at two levels 1.5% CDT and 3.8% CDT. Within run CVs were 2.4% and 2.2% (n=10), and 4.3% and 3.5% (n=12) between run respectively.

4.2.3 Histopathological analysis and biochemical studies

Liver biopsies were fixed in 10% buffered formalin, embedded in paraffin, and standard 5μm sections prepared and stained with haematoxylin-eosin. The extent of fibrosis was assessed by a specialised liver histopathologist (AC) using a modified METAVIR score, (modified from[163]) which was performed as follows: stage 1, portal or central fibrosis; stage 2, some septa; stage 3, many septa; stage 4, cirrhosis. The METAVIR scoring system was used to assess hepatic inflammation[162]. Steatosis was graded as follows: 0 (<5% hepatocytes affected); 1 (5-33% of hepatocytes affected); 2 (34-66% of hepatocytes affected); or 3 (>66% of hepatocytes affected). Iron stores were graded 0-4 following Perls' staining.

Routine haematology and biochemical tests were recorded if performed within 5 weeks of the liver biopsy. These tests included γ-GT, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and MCV.

4.2.4 Statistical Methods

Statistical analyses were performed in GraphPad/Prism (version 5.03) and utilized the analysis of variance (ANOVA), student's t-test, Mann-Whitney U test, and Spearman's non-parametric correlation co-efficient where appropriate. A p value of <0.05 was considered significant.

4.3 Results

4.3.1 Patient characteristics

The demographic characteristics of patients are detailed in the Table 4.1. The most common causes of liver disease were chronic hepatitis C (HCV) (113, 44.5% of subjects), hepatitis B (HBV) (64, 25.2%) and NAFLD (42, 16.5%). 18 of the 254 patients had a diagnosis of ALD, although only 6 reported heavy alcohol consumption at the time of liver biopsy. Another 6 subjects acknowledged heavy drinking as a co-factor with chronic HCV (n=5) or HBV (n=1). Overall, the mean age of subjects was 45.4 years and the M:F ratio was

approximately 2:1 (172 (67.7%) were male). 48 patients (18.9%) had cirrhosis as determined by liver biopsy. BMI was lean in 75 patients (29.5%), overweight in 87 (34.3%) and obese in 86 (33.9%). BMI was not determined in 6 subjects (2.3%).

Table 4.1. Demographic and histological details of the 254 subjects at the time of liver biopsy

		Alcohol Group[#]			
	All (n)	**0**	**1**	**2**	**3**
Subjects (n)	254	104	125	13	12
Caucasian (%)	72	59	78	100	100
Age (years) (mean ± SD)	45.4 (11.0)	45.4 (11.2)	44.9 (11.3)	50.1 (8.6)	46.2 (8.44)
BMI (kg/m^2) (mean ± SD)	27.4 (5.4)	27.5 (6.2)	27.2 (4.7)	29.3 (6.4)	25.4 (4.1)
Smoker (%)	40.0	38.5	34.4	46.2	83.3
Male (%)	68	58	75	54	92
HBV (n)	64	36	27	0	1
HCV (n)	113	39	59	10	5
Autoimmune (n)	7	3	4	0	0
NAFLD (n)	42	16	26	0	0
ALD (n)	18	3	6	3	6
Other* (n)	10	7	3	0	0
Stage of fibrosis					
0 (n)	45	17	24	2	2
1 (n)	62	27	31	3	1
2 (n)	54	18	28	5	3
3 (n)	45	16	21	3	5
4 (n)	48	26	21	0	1
Grade of steatosis					
0 (n)	80	34	41	3	2
1 (n)	88	40	41	7	0
2 (n)	48	15	27	1	5
3 (n)	38	15	16	2	5

Table 4.1 notes. [#]Subjects were grouped according to alcohol consumption: Group 0: 0 g/wk; Group 1: females >0-140 g/wk; males >0-210 g/wk; Group 2: females >140-<350 g/wk, males >210-<420 g/wk; Group 3: females ≥ 350 g/wk, males ≥ 420 g/wk. *Other: drug induced liver injury (n=3), seroconverted HBV (n=1), abnormal liver enzymes associated with diabetes mellitus/thyroid disease (n=3), Wilson's disease (n=1), non-diseased liver (n=2). *Abbreviations: BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD, non-alcoholic fatty liver disease; ALD, alcohol-related liver disease.*

In the subjects drinking alcohol, median reported alcohol consumption within 2 weeks of liver biopsy was 45 (0.2-1505) g/week (104 abstained) and median reported alcohol intake 6 months prior was 42.5 (0.2-1505) g/week (91 abstained). Subjects were classified into the following alcohol groups based on nurse interviews at the time of liver biopsy: Group 0, no alcohol consumption; Group 1, less than recommended maximum weekly allowance (females >0-140g/week, males >0-210g/week); Group 2, greater than recommended maximum weekly allowance but less than heavy (females >140-<350g/week, males >210-<420g/week); Group 3, heavy (females ≥350g/week, males ≥420g/week). Few patients (n=12) acknowledged drinking heavily at the time of liver biopsy. All were Caucasian and the majority were male, with HCV or alcohol-related liver disease.

4.3.2 CDT assay

Figure 4.1 illustrates a characteristic HPLC chromatogram of a serum sample from a) a subject drinking less than recommended weekly allowance and b) a heavy drinker. The HPLC peaks representing different transferrin glycoforms were readily identified from their positions in the chromatogram, and illustrate an increase in disialotransferrin in the heavy drinker. Disialo- and trisialotransferrin were efficiently separated in all 254 patients, despite previous reports that di-tri bridging occurs in cirrhotic patients[132].

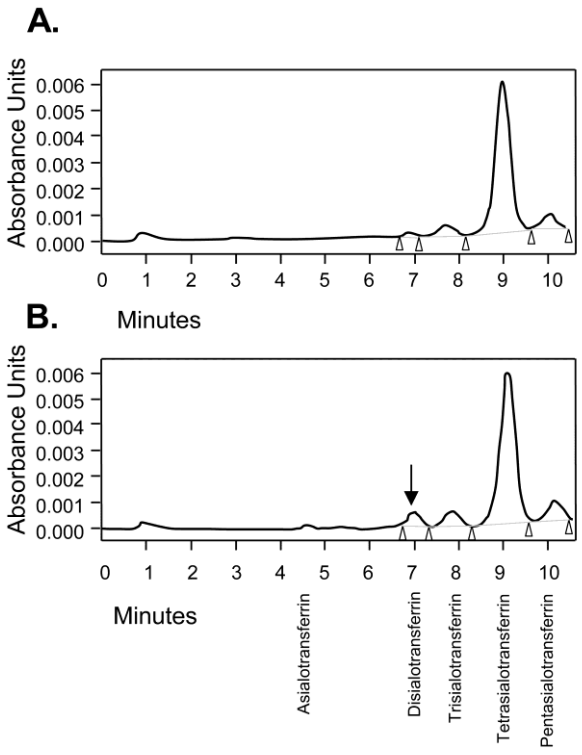

Figure 4.1. CDT assay. HPLC chromatographs depicting carbohydrate-modified serum transferrin isoforms (**A**) Normal serum transferrin profile. (**B**) carbohydrate deficient transferrin profile showing increased disialotransferrin (arrow).

4.3.3 Characteristics of patients with %CDT >1.7

The laboratory reference value indicative of heavy drinking is %CDT >1.7[118]. Of the 254 subjects, only 13 had a %CDT >1.7, predictive of heavy drinking. 7 of these 13 subjects acknowledged heavy drinking, and 6 reported drinking less than 350 grams of alcohol per week over the prior 2 weeks. However, review of their medical records, other blood markers (serum ferritin, MCV, γ-GT) and liver histology, suggested that 5 of these 6 subjects were actually heavy drinkers. The remaining subject had consumed excessive alcohol for many years, but had reportedly reduced his intake prior to the liver biopsy.

4.3.4 %CDT is not affected by stage or aetiology of non-alcoholic liver disease

Previously it has been reported that stage or aetiology of liver disease may affect %CDT[129,132,133]. In our patient cohort, no significant difference in %CDT was noted in relation to gender, smoking, age or ethnicity. Importantly there was no significant difference in %CDT result according to liver fibrosis stage (Figure 4.2A), or aetiology of liver disease, except in subjects with alcohol-related liver disease (Figure 4.2B).

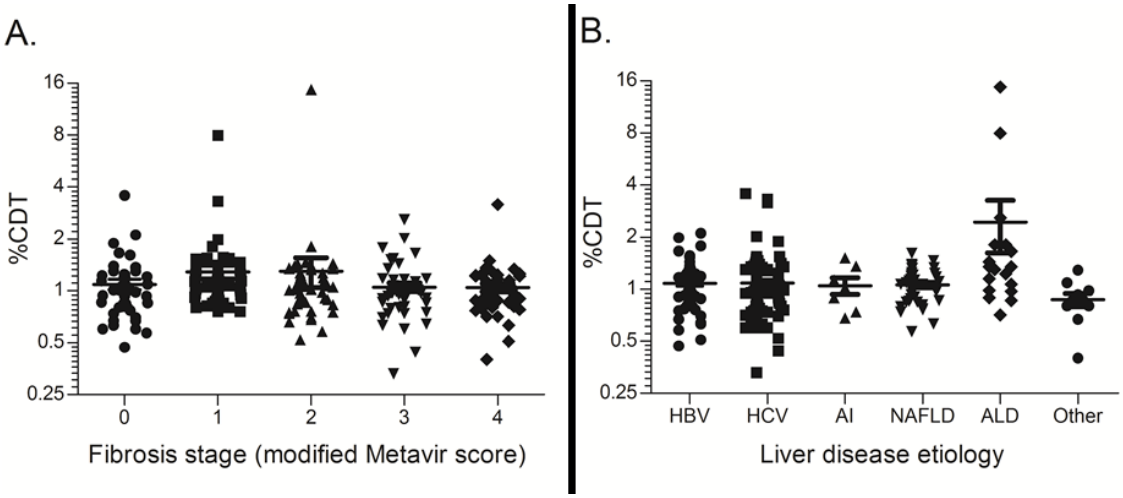

Figure 4.2. %CDT is not affected by stage or aetiology of non-alcoholic liver disease. %CDT in patients at different stages (**A**) or with different aetiologies of liver disease (**B**). *Abbreviations: HBV, hepatitis B; HCV, hepatitis C; AI, autoimmune liver disease; NAFLD, non-alcoholic fatty liver disease; ALD, alcohol-related liver disease.*

4.3.5 Characteristics of patients with heavy alcohol consumption and %CDT ≤1.7: BMI affects %CDT

Five of the 12 acknowledged heavy drinkers had %CDT below the cut off despite documented chronic excessive alcohol use and sustained consumption of >50-80 g/day for at least 2 weeks. Furthermore, these 5 subjects had liver steatosis and other blood markers (increased γ-GT and MCV) in keeping with heavy drinking. In the cohort of subjects with an elevated %CDT or acknowledged heavy alcohol consumption (females ≥350g/week, males ≥420g/week), the only clinical variable significantly associated with %CDT was BMI, with %CDT lower in overweight or obese subjects compared with lean. (Figure 4.3A, two-tailed Mann Whitney U test P=0.004). Alcohol consumption and %CDT were correlated in drinkers, but the correlation was better for lean (r_s=0.51, P<0.001) than overweight subjects (r_s=0.22, P=0.004) (Figure 4.3B).

Considering the effects of body size and composition on alcohol concentrations, alcohol consumption was corrected for apparent volume of distribution of ethanol (Vd) using estimated lean body weight (LBW) as a surrogate for Vd. In subjects with CDT>1.7% and/or acknowledged heavy drinking, mean alcohol consumption per estimated Vd (g/week/LBW) was similar in lean and overweight subjects (Figure 4.3C, P=0.13, unpaired t-test) and did not account for the lower %CDT in overweight heavy alcohol consumers. (Figure 4.3D) Overweight heavy drinkers with CDT≤1.7% were consuming similar amounts of alcohol per estimated Vd to lean drinkers with CDT>1.7%. When corrected for lean body weight, the correlation for alcohol consumption and %CDT in lean drinkers was r_s=0.63, p<0.001, whereas for overweight drinkers it was r_s=0.18, P=0.08.

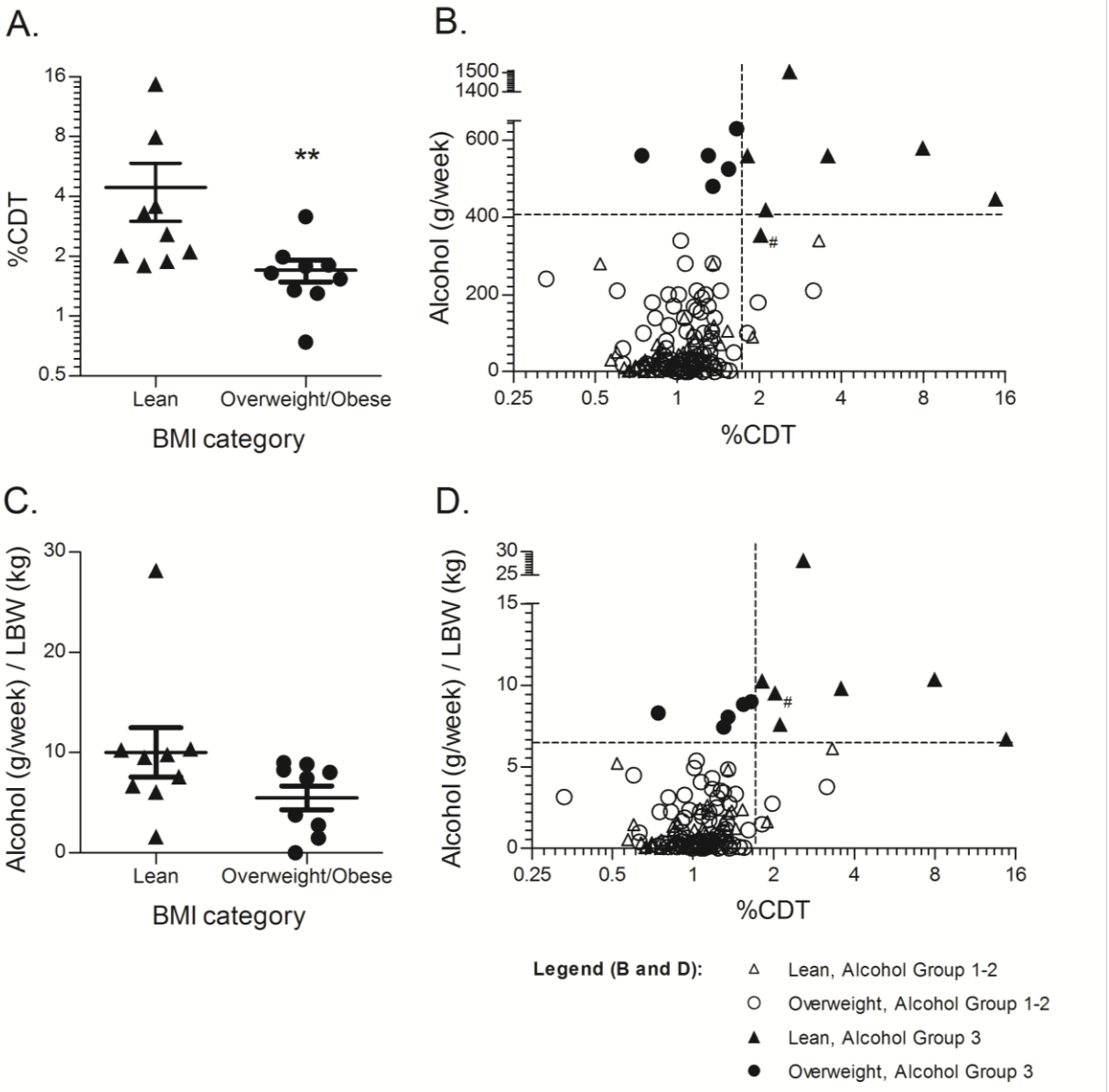

Figure 4.3. BMI affects %CDT. (**A**) %CDT in lean and overweight/obese patients who acknowledged heavy drinking, or whose %CDT was >1.7 (**p<0.005) (**B**) Alcohol consumption vs %CDT in lean and overweight drinkers, highlighting Alcohol Group 3 (horizontal line represents the male heavy drinking threshold (>420g/week), # is female). Vertical line represents 1.7% CDT (**C**) Alcohol consumption corrected for apparent volume of distribution of ethanol in subjects with CDT>1.7% and/or who acknowledged heavy drinking (**D**) Alcohol consumption corrected for volume of distribution vs %CDT in lean and overweight drinkers. The horizontal line demarcates Alcohol Group 3. Vertical line represents 1.7% CDT.

4.3.6 %CDT is a useful marker of alcohol abuse in lean liver disease patients, but not of alcohol use

Although %CDT correlated with alcohol consumption amongst the drinking population (Figure 4.3 B,D), its utility to distinguish subjects drinking lower amounts of alcohol or abstaining was compromised by the broad distribution of %CDT in (self-reported) non-heavy drinkers (Figure 4.4).

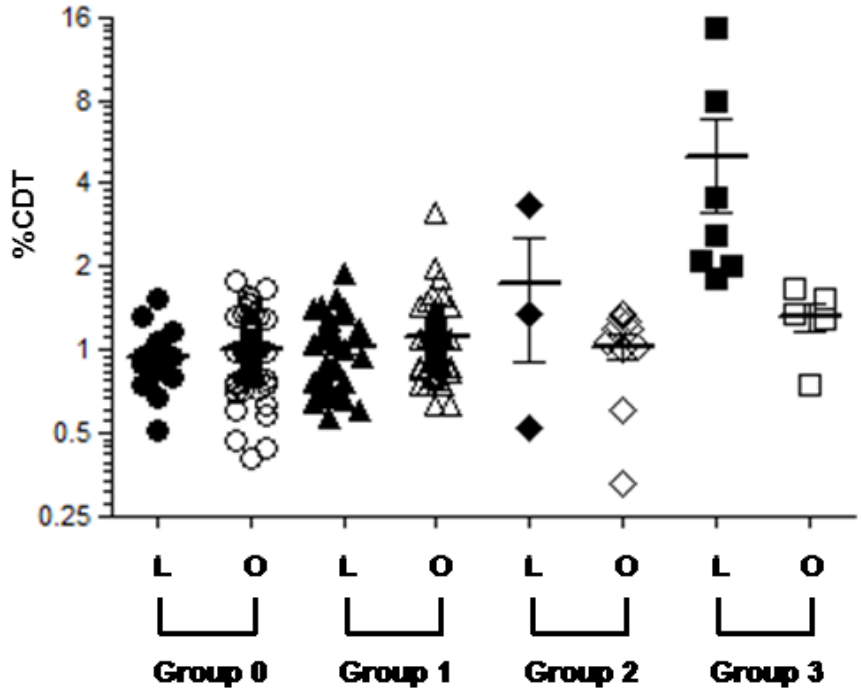

Figure 4.4. %CDT is a useful marker of alcohol *abuse* in lean liver disease patients but not of alcohol use. %CDT in lean (L) and overweight (O) patients with chronic liver disease grouped according to alcohol consumption, including abstainers (Group 0).

4.4 Discussion

CDT is reported to be the most specific marker of alcohol abuse[119] and its clinical utility has improved with the introduction of a standardised analytical technique using HPLC. This study was undertaken to determine whether %CDT, as determined by the standardised assay, is an accurate marker of heavy alcohol consumption in patients with chronic liver disease, or whether clinical variables such as BMI or disease aetiology interfere with %CDT estimation. The HPLC-based %CDT assay is reported to be 95% specific for heavy drinking if levels are >1.7[118]. In our cohort of 254 subjects, 13 had a %CDT >1.7, predictive of heavy alcohol intake at the time of liver biopsy, 6 of whom did not acknowledge heavy drinking. 12 of these 13 subjects were suspected current heavy drinkers on review of their medical records and supportive biochemical and haematological laboratory tests. An elevated %CDT may therefore be a useful laboratory finding to strengthen or confirm a clinical suspicion of alcohol abuse.

In our series of patients, the presence of chronic liver disease did not influence interpretation of the CDT results. This is in contrast to earlier reports of poor chromatographic resolution of disialotransferrin from trisialotransferrin[132,133]. Di-tri bridging does not allow accurate quantification of disialotransferrin due to suboptimal separation from trisialotransferrin and may lead to “false positive” results if not recognized and interpreted correctly. The mechanism underlying poor chromatographic resolution of transferrin glycoforms in these earlier studies is not clear, although it has been noted that abnormal protein glycosylation occurs in liver disease[470-472]. The reason for the complete *absence* of di-tri bridging in our well-characterized patient cohort that included 48 subjects with cirrhosis remains unclear. All of our patients had well-compensated chronic liver disease and it is possible that di-tri bridging may only occur in patients with decompensated disease.

Although we were confident that subjects with CDT>1.7% were drinking heavily; the subjects who acknowledged heavy drinking at the time of liver biopsy, but did not exhibit CDT>1.7% were of concern. Heavy drinkers with a BMI in the overweight or obese range had significantly lower %CDT than heavy drinkers in

the normal weight range. This is a critical observation that may reduce the diagnostic utility of %CDT. Two previous studies recruited from a large twin registry identified that an “insulin resistant” phenotype (increased BMI and triglyceride levels, low HDL cholesterol) was associated with reduced effect of alcohol intake on the CDT response[123,125]. Similarly, a study of hypertensive men with metabolic risk factors demonstrated a positive relationship between serum CDT concentration and glucose disposal rate during hyperinsulinemic euglycemic clamp[124]. In these studies, serum CDT was determined by immunoassay tests (RIA or immunonephelometric assay) and relatively few subjects were consuming >280 g alcohol/week. Our findings confirm and then build on this important observation, showing that an elevated BMI reduces the diagnostic utility of CDT at higher alcohol intake in subjects with chronic liver disease using the standardized HPLC method.

The mechanisms responsible for increased serum CDT in response to alcohol abuse and the influence of BMI on this response remain unclear. A decrease in glycosyltransferases involved in transferrin carbohydrate side chain synthesis and an increase in sialidase activities were found in human subjects with alcohol abuse compared with nondrinking subjects[473]. Similar results were obtained in alcohol-treated rats and were attributed to a direct effect of alcohol or its oxidation product, acetaldehyde[473]. Loss of carbohydrate residues may also lead to uptake of the altered protein by different carbohydrate-specific receptors or altered affinity for the receptor, although relatively little is known about the process and kinetics of CDT’s elimination from the circulation. Clearance of senescent glycoproteins which are deficient in terminal sialic acids occurs via the hepatocyte asialoglycoprotein receptor[474] and there is some evidence that activity of this receptor may be modulated by variation in insulin and glucose levels[475,476].

In our cohort of subjects with chronic liver disease, the threshold for appearance of %CDT>1.7 appears similar to the published data, requiring heavy alcohol consumption of at least 350 g/week[469]. %CDT correlated with alcohol consumption in drinkers, but the correlation was better in normal weight drinkers than overweight drinkers. Adjusting alcohol consumption for gender and

apparent volume of distribution of alcohol by using lean body weight did not change the correlation for overweight drinkers. This implies that the decreased %CDT in overweight drinkers is not simply due to a larger volume of distribution of ethanol. This is not necessarily surprising, as the liver is a (if not the) major site of first-pass alcohol metabolism, in addition to being exposed to circulating blood alcohol. Nevertheless, whilst correcting for volume of distribution of ethanol may not explain the reduced CDT response in overweight drinkers, it may allow a more accurate individual assessment of ethanol concentrations that should be considered ‘heavy’ in lean drinkers. Despite the positive correlation between %CDT and alcohol consumption, there was a broad distribution of CDT in non-drinkers, confirming its inability to distinguish subjects drinking lower amounts of alcohol. In a hepatology outpatient setting, this assay is likely to be useful to confirm suspicion of heavy drinking in subjects who are not overweight, but cannot reliably identify moderate drinkers.

CHAPTER 5

DIAGNOSTIC PERFORMANCE OF CARBOHYDRATE DEFICIENT TRANSFERRIN IN HEAVY DRINKERS

Fagan KJ[1,2], Irvine KM[2], McWhinney BC[4], Fletcher LM[1,3], Horsfall LU[1,2], Johnson LA[4], O'Rourke P[5], Martin J[3,6], Scott I[6], Pretorius C[4], Ungerer JP[4], Powell EE[1,2]. Diagnostic sensitivity of carbohydrate deficient transferrin in heavy drinkers. *BMC Gastroenterol*. 2014; **14**:97.

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Contributor	Statement of contribution
Kevin Fagan *DM candidate*	Conceived and designed study (80%) Collected data and samples (90%) Data analysis (55%) Wrote the paper (100%) Edited the paper (20%)
Katharine Irvine *Research fellow*	Data analysis (15%) Edited the paper (20%)
Brett McWhinney *Scientist*	Performed CDT analysis (45%)
Linda Fletcher *Scientist*	Edited the paper (5%)
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Abstract

Background and Aim: Carbohydrate deficient transferrin (CDT) is the most specific serum biomarker of heavy alcohol consumption, defined as ≥350–420g alcohol/week. Despite introduction of a standardized reference measurement technique, widespread use of CDT remains limited due to low sensitivity. The aim of this study was to determine the factors that affect diagnostic sensitivity in patients with sustained heavy alcohol intake.

Methods: Patients with a self-reported history of sustained heavy alcohol consumption were recruited from the hepatology outpatient department or medical wards. Each patient was interviewed with a validated structured questionnaire of alcohol consumption and CDT analysis using the standardized reference measurement technique with high performance liquid chromatography was performed on serum collected at time of interview.

Results: 52 patients were recruited: 19 from the hepatology outpatient department and 33 from general medical wards. Median alcohol intake was 1013 (range 366-5880) g/week over the preceding two week period. 26 patients had a diagnostic CDT based on a threshold value of %CDT >1.7 indicating heavy alcohol consumption, yielding a sensitivity of 50%. Overweight/obesity (defined as body mass index (BMI) ≥25 kg/m^2 in Caucasians and ≥23.0 kg/m^2 in Asians), female gender and presence of cirrhosis were independently associated with non-diagnostic %CDT (≤1.7).

Conclusions: CDT has limited sensitivity as a biomarker of heavy alcohol consumption. Caution should be applied when ordering and interpreting %CDT results, particularly in women, patients with cirrhosis and those with an elevated BMI.

5.1 Introduction

The relative amount of serum carbohydrate-deficient transferrin (CDT) is currently the most specific serum biomarker of heavy alcohol consumption[119]. CDT refers to a temporary alteration in the glycosylation pattern of transferrin resulting in an increase in the relative amounts of disialo- and asialo-transferrin (and a decrease in tetrasialotransferrin) that occurs as a result of sustained heavy alcohol consumption (thresholds range from 50-80g of alcohol/day for at least 2 weeks). Altered transferrin glycosylation patterns return to baseline levels within 2 to 5 weeks following complete abstinence from alcohol[117]. Using the standardized reference measurement technique with high performance liquid chromatography (HPLC) and quantification of disialotransferrin as a percentage of total transferrin (%CDT), a value of >1.7 is considered to be specific for sustained heavy alcohol consumption[118]. Very few circumstances are associated with “false-positive” %CDT results using HPLC. These include genetic transferrin variants[115], rare congenital disorders of glycosylation[120] and pregnancy[121,122].

In contrast to the high specificity, diagnostic sensitivity of %CDT for detection of heavy alcohol intake is low. Previous studies using older methods of CDT analysis such as immunoassays and anion-exchange methods have identified several patient characteristics that affect diagnostic sensitivity[123-128]. These characteristics include gender and metabolic risk factors such as obesity, insulin resistance, hypertension and dyslipidemia. We recently examined the diagnostic utility of %CDT in a hepatology outpatient setting[454]. Although few patients reported heavy alcohol consumption at the time of study, those acknowledged heavy drinkers with a body mass index (BMI) in the overweight or obese range had significantly lower %CDT values than lean heavy drinkers[454]. Neither the presence of compensated chronic liver disease, nor the etiology of non-alcoholic liver disease influenced interpretation of the CDT results. A key limitation of our earlier study and other previous studies investigating %CDT is the inclusion of patients with a broad range of alcohol intake and a relatively small proportion of patients with a heavy alcohol intake, at a level expected to cause %CDT >1.7.

Despite recognition that clinical history and self-report screening tests are efficient methods to identify at-risk patients, there is clearly a need for an objective biomarker to support clinical suspicion of heavy alcohol intake. In order to improve the clinical utility of CDT measurements, factors that affect the diagnostic sensitivity and specificity need to be clearly defined, so that the test is requested and interpreted appropriately. The aim of this study was to determine in patients with *sustained heavy alcohol intake*, whether the level of %CDT is influenced by BMI or other clinical variables such as gender, age, ethnicity and smoking. To our knowledge, this is the first time that these factors have been examined in a cohort of patients with sustained heavy alcohol consumption.

5.2 Materials and Methods

5.2.1 Patients and clinical data

Patients with self-reported heavy alcohol consumption were recruited from the hepatology outpatient department or medical wards at the Princess Alexandra Hospital, Brisbane, Australia during 2012 and 2013. Informed written consent was obtained from each patient and the protocol was approved by Metro-South-Health and the University of Queensland Human Research Ethics Committees. Those who agreed to participate were interviewed by the research co-ordinator using a structured questionnaire and a standard drink guide.

The questionnaire included an alcohol calendar to record alcohol consumption over the prior 4-week period and further direct questions to determine whether the calendar reflected usual alcohol consumption. It also recorded any previous periods of heavy alcohol consumption, defined as ≥350g/week for females and ≥420g/week for males for ≥6 months. These questions were supplemented by validated alcohol screening tools; the Alcohol Use Disorders Identification Test (AUDIT)[28] and the Brief Michigan Alcoholism Screening Test (bMAST) [47], to confirm current heavy alcohol consumption (as previously defined) and identify alcohol dependence.

Measurements of weight and height were obtained from patients at the time of interview. BMI was calculated as weight in kg/(height in meters)^2. BMI was classified as lean (<25 kg/m^2 in Caucasians, <23 kg/m^2 in Asians), overweight (25-29.9 kg/m^2 in Caucasians, 23.0 to 24.9 kg/m^2 in Asians) or obese (≥30 kg/m^2 in Caucasians, ≥25.0kg/m^2 in Asians). Lean body weight (LBW) was calculated using the Janmahasatian equation, as this has been validated in an obese population[467], and then used to estimate the volume of distribution (Vd) of alcohol, since fat has little water.

The medical record was reviewed to ascertain demographic details, previously diagnosed liver disease and other medical conditions, medications and history of alcohol, tobacco and recreational drug use. Standard biochemical and serological assays, liver imaging and histological assessment of a liver biopsy (if performed) were used to assess diagnosis and etiology of liver disease. In the absence of a liver biopsy, cirrhosis was determined on the basis of a Fibroscan® result >14 kPa[242,477] and/or liver imaging (nodular or irregular liver surface and/or features of portal hypertension) in conjunction with other clinical and/or biochemical parameters. The severity of liver disease was evaluated using the Child-Turcotte-Pugh (CTP) classification. All patients with chronic hepatitis C had detection of circulating HCV RNA by polymerase chain reaction using the Abbott m2000 RealTime System (Abbott Laboratories, Illinois, USA). Routine haematological and biochemical tests were performed within 1-3 days of interview and serum collection for CDT analysis.

5.2.2 CDT analysis

Serum was collected at the time of interview and stored at -80ºC, a condition under which the transferrin isoform pattern is stable[468]. CDT analysis was performed on a Waters HPLC System (Waters Corporation Milford MA USA) as previously described[454]. The currently accepted laboratory reference value indicative of heavy drinking is %CDT >1.7[118].

5.2.3 Statistical Methods

Statistical analyses were performed in SPSS, employing Fisher's exact test for categorical variables, either t-test or Mann-Whitney U-test for continuous

variables and Spearman correlation analysis for univariate tests. Logistic regression with backward elimination of non-significant terms was used for multivariate models. A p-value of <0.05 was considered statistically significant.

5.3 Results

5.3.1 Patient characteristics

Overall, 19 patients were recruited from the hepatology outpatient department and 33 were approached within 48 hours of admission to a general medical ward. All 52 patients reported previous periods of heavy alcohol consumption and excessive alcohol use during the 4 weeks prior to interview, with a median intake of 1013 (range 366-5880) g/week over the preceding 2 week period. In the general medicine group, the reason for presentation was: alcohol intoxication/withdrawal symptoms (n=21), alcoholic hepatitis (n=4), gastrointestinal bleed (n=3), infection (n=4) and pancreatitis (n=1). Overall, the mean age of subjects was 50.3 (±11.8) years, 37 (71.2%) were men and 45 (86.5%) were Caucasian. BMI was lean in 27 patients (51.9%), overweight in 12 (23.1%), and obese in 13 (25%).

Eighteen patients (34.6%) had cirrhosis as determined by liver biopsy or imaging and 15 patients had evidence of concurrent hepatitis C infection (HCV). Other chronic medical conditions included: type 2 diabetes (n=6), hypertension (n=20), hyperlipidaemia (n=9), rheumatoid arthritis (n=2), COPD/asthma (n=9), chronic kidney disease (CKD) ≥stage 3 (estimated glomerular filtration rate (eGFR) ≤59) (n=2).

5.3.2 Characteristics of patients with %CDT ≤ or >1.7

Despite all 52 patients demonstrating heavy drinking based on results of questionnaires, only 26 had a %CDT >1.7. The characteristics of patients with %CDT ≤ or >1.7 are detailed in Table 5.1. A statistically significant difference in BMI was seen between heavy drinkers with a “diagnostic” or “non-diagnostic” %CDT. The mean (+/- SD) BMI of heavy drinkers with %CDT >1.7 was 23.3 (+/- 3.9) kg/m^2, with 73.1% within the lean weight range. In contrast, the mean (+/-

SD) BMI for heavy drinkers with %CDT ≤1.7 was 28.2 (+/- 7.2) kg/m^2, with only 30.8% within the lean weight range. Eighteen of 25 patients (72%) with BMI in the overweight/obese range had %CDT ≤1.7. The two overweight/obese patients with notably raised %CDT had CKD stage 3, with moderately reduced kidney function (eGFR 30-59). The presence of hypertension did not differ in relation to %CDT ≤ or >1.7. Diabetes and hyperlipidemia were infrequent comorbidities in this group of patients and therefore their impact could not be evaluated.

Table 5.1. Demographic and clinical details of patients in relation to the %CDT reference cut-off value of 1.7.

	%CDT ≤1.7	**%CDT >1.7**	**P-value**
Subjects (n)	26	26	
Caucasian (n, %)	22 (84.6)	23 (88.5)	1.00
Age (years) mean (± SD)	51.1 (±10.2)	49.6 (±13.3)	0.67
Gender (n, % men)	13 (50.0)	24 (92.3)	0.002
BMI (kg/m^2) mean (± SD)	28.2 (±7.2)	23.3 (±3.9)	0.003
Smoker (n, %)	14 (53.8)	20 (76.9)	0.14
Median alcohol consumption last 2 weeks (g/week) (range)	868 (366-2100)	1258 (510-5880)	0.005
Median estimated alcohol/Vd (g/week/kg) (range)	17.3 (6.7-42.2)	24.1 (7.4-82.5)	0.007
AUDIT mean (± SD)	27.6 (±7.2)	28.7 (±6.9)	0.57
bMAST mean (± SD)	17.6 (±8.1)	22.5 (±6.2)	0.018
Cirrhosis (n, %)	15 (57.7)	3 (11.5)	0.001

Fifteen of 18 patients (83.3%) with cirrhosis had a non-diagnostic %CDT. Of these 15 patients, 7 had compensated disease (CTP score A), 7 had functional compromise (CTP score B) and 1 had decompensated liver disease (CTP score C). The 3 cirrhotic subjects with %CDT >1.7 had compensated disease (CTP score A). A statistically significant difference was also seen between gender and %CDT category, with women far less likely than men, to have a diagnostic %CDT. In contrast, ethnicity, age, and smoking status were comparable between the %CDT categories.

Median alcohol consumption over the 2 weeks prior to interview was higher for patients with %CDT >1.7 (1257.5 g/week) compared to subjects with %CDT ≤1.7 (867.5 g/week; $p<0.005$) (Table 5.1). To consider the effects of body size and composition on alcohol concentrations, alcohol consumption was corrected for apparent volume of distribution (Vd) of alcohol using estimated lean body weight (LBW) as a surrogate for Vd. Median alcohol consumption per estimated Vd was 17.3 and 24.1 g/week/kg LBW in patients with %CDT ≤ and >1.7 respectively ($P<0.007$) (Table 5.1). Alcohol consumption (g/wk/kg LBW) and %CDT were correlated, but the correlation was better for lean ($r_s=0.51$, $P<0.01$) than overweight subjects ($r_s=0.18$, $P=0.40$), non-cirrhotic ($r_s=0.54$, $P<0.001$) compared with cirrhotic subjects ($r_s=0.02$, $P=0.94$) and males ($r_s=0.48$, $P<0.01$) compared with females ($r_s=0.15$, $P=0.60$) (Figure 5.1).

Selected laboratory data of patients in relation to the %CDT reference cut-off value of 1.7 are detailed in Table 5.2. No statistically significant differences between the two groups for laboratory tests commonly used in clinical practice to suggest sustained heavy alcohol use (serum aminotransferases, gamma-glutamyltransferase, platelet count and mean corpuscular volume) were seen.

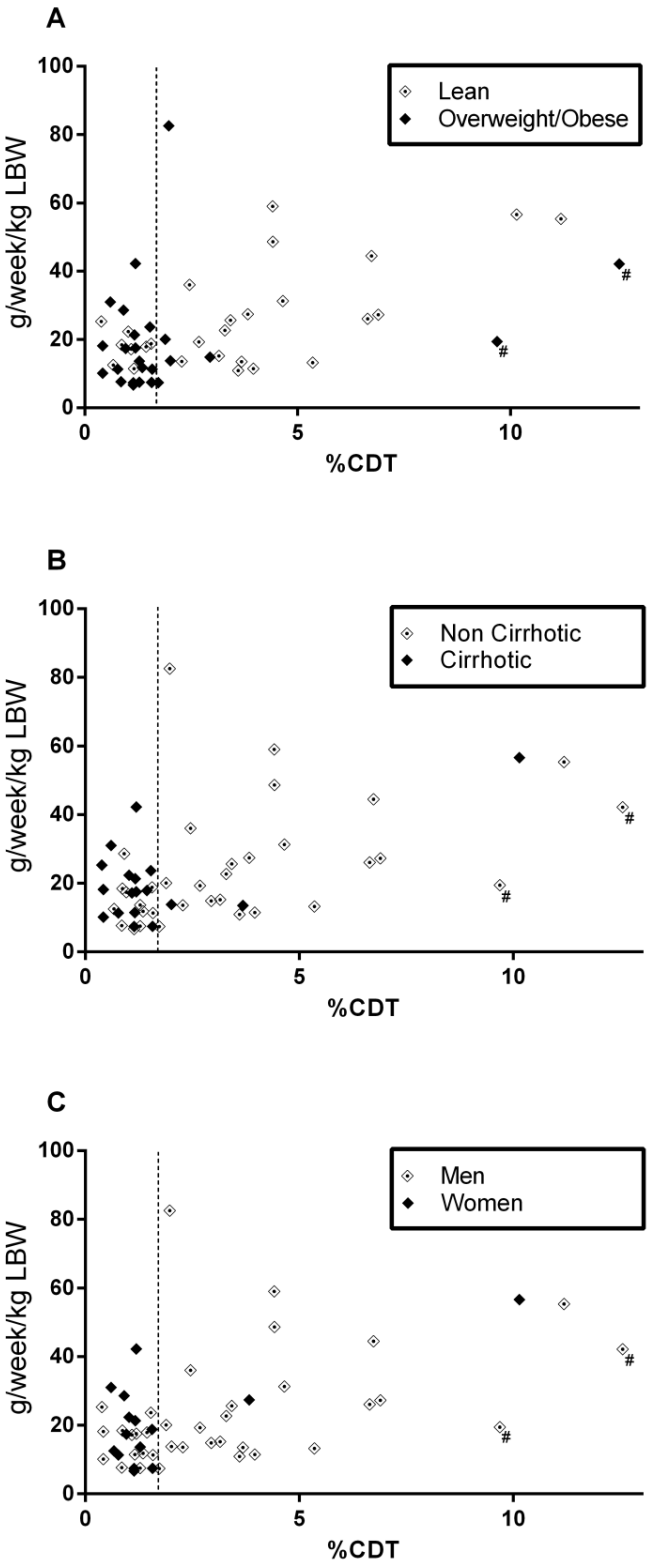

Figure 5.1. Correlation of alcohol consumption (g/wk/kg LBW) and %CDT for: (**A**) lean vs. overweight/obese subjects; (**B**) non cirrhotic vs. cirrhotic subjects; and (**C**) men vs. women. (# identifies the 2 patients with moderately decreased renal function (eGFR 30-59)). (LBW = Lean body weight).

Table 5.2. Selected laboratory data of patients in relation to the %CDT reference cut-off value of 1.7.

Laboratory test, median (interquartile range)	**Normal range**	**%CDT ≤1.7**	**%CDT >1.7**	**P-value**
Alkaline phosphatase (U/L)	*53-128*	101.5 (77.0-161.5)	90.5 (72.8-106.3)	0.16
Gamma-glutamyl transferase (U/L)	*<55*	182.0 (110.0-513.3)	135.5 (43.8-283.3)	0.09
Alanine aminotransferase (U/L)	*<45*	54.0 (26.0-89.0)	57.5 (28.0-93.5)	0.98
Aspartate aminotransferase (U/L)	*<35*	100.0 (49.0-163.5)	76.0 (47.0-139.8)	0.41
Platelets (x 10^9/L)	*140-400*	165.5 (94.3-209.3)	168 (130.5-211.5)	0.37
Mean cell volume (fL)	*80-100*	98.5 (95.8-101.5)	96.5 (90.8-100.3)	0.16

Following multivariate analysis initially including age, gender, cirrhosis, BMI category, alcohol consumption and smoking status, overweight/obesity (OR=5.8, p=0.047), presence of cirrhosis (OR=17.2, p=0.007), female gender (OR=14.3, p=0.019) and lower alcohol consumption (OR=0.998, p=0.029) remained independently associated with %CDT ≤1.7. (Table 5.3)

Table 5.3. Variables independently associated with a non-diagnostic %CDT identified by logistic regression. For categorical variables, the odds ratio refers to the category shown in brackets; for alcohol consumption the odds ratio refers to the decreased likelihood of a non-diagnostic %CDT per increase in alcohol consumption by 1g/week.

		95% Confidence Intervals		
Variable	**Odds Ratio**	**Lower**	**Upper**	**P-value**
Gender (Women)	14.3	1.5	132.0	0.019
BMI (Overweight/Obese)	5.8	1.0	32.9	0.047
Cirrhosis (Yes)	17.2	2.2	137.0	0.007
Alcohol consumption over prior 2 weeks (g/week)	0.998	0.996	1.000	0.029

5.4 Discussion

Although %CDT (determined by the HPLC assay) remains the most specific serum biomarker of prolonged heavy alcohol consumption[119], its widespread use in clinical practice remains limited, largely due to concern about poor sensitivity and uncertainty about the factors that impact on CDT response to alcohol. This study was undertaken to identify clinical variables that affect the sensitivity of the standardized HPLC-based CDT assay in detecting heavy drinkers. Our study shows that only 50% of subjects drinking >50-60g alcohol daily for at least 2 weeks had a %CDT >1.7%, indicative of heavy alcohol intake. Overweight/obesity, the presence of cirrhosis and female gender were independently associated with a non-diagnostic %CDT level (≤1.7).

Previous population-based studies measuring CDT by ion-exchange chromatography and immunoassay found several patient characteristics, including gender, a high BMI and an insulin-resistant phenotype (high triglycerides and low HDL-cholesterol) were associated with reduced sensitivity

of the CDT response to alcohol[123,125]. In contrast, more recent studies that quantified CDT using the standardized HPLC method did not find any clinically significant differences in CDT in relation to gender or BMI[130]. The authors concluded that the earlier findings were related to the analytical techniques used for measurement of CDT, and that adjustment of reference intervals in relation to gender or BMI was not required [118,130]. However, a major limitation of these studies was the low or unclear number with confirmed heavy drinking. In our study involving only confirmed heavy drinkers, elevated BMI and female gender clearly reduce the diagnostic sensitivity of %CDT using the standardized HPLC method.

Reporting CDT as relative amount of total transferrin concentration rather than an absolute value has improved sensitivity and specificity of the assay[478]. Introduction of this method was expected to negate many of the factors attributed to gender (e.g. pregnancy, oestrogens and anaemia), since they can cause variations in total transferrin concentrations. However, recent reports using %CDT have demonstrated that gender differences[479] and pregnancy-related changes in CDT isoform levels occur, although no biologic mechanism has been described[122,480]. Women may differ in the CDT isoforms that are increased by heavy alcohol intake, such as asialo- and monosialotransferrin[114], neither of which are included in %CDT measurement using the new standardised HPLC technique. This would be in keeping with previous findings that women express higher CDT levels under basal conditions, but produce less in response to heavy drinking[134,481].

We previously investigated the diagnostic utility of %CDT in patients with liver disease, and found that heavy drinkers with a BMI in the overweight or obese range had significantly lower %CDT values than lean heavy drinkers[454]. The current study extends these findings by confirming the results in a larger group of subjects with confirmed heavy alcohol consumption and by showing that the effect of BMI is independent of other clinical variables. Interestingly 2 subjects had markedly elevated %CDT values (9.68% and 12.55%) despite overweight/obesity, in the setting of moderately decreased renal function (eGFR 30-59). Currently little is known regarding the process and elimination kinetics of

CDT from the circulation and thus the mechanisms responsible for this effect are unclear, but may relate to altered elimination in the presence of renal failure[482]. Chronic kidney disease does not appear to cause an increase in the baseline levels of CDT in subjects without hazardous drinking[483]. Similarly, non-enzymatic glycation of transferrin, a process that may occur in uremia[482] and diabetic subjects[484] does not appear to interfere with HPLC-based CDT measurement[485].

In our prior study we found that the presence of cirrhosis due to various chronic liver diseases did not lead to “false positive” %CDT results[454]. In the current study of heavy drinkers, cirrhosis was associated with *reduced* sensitivity of the %CDT response to alcohol, which is contrary to some previous reports[132,133,486]. This finding confirms earlier studies using non-HPLC methods that found patients with cirrhosis and a high current alcohol intake had lower CDT values compared with “control” subjects without liver disease but drinking more than 50g alcohol/day[487]. The reasons underlying these findings remain unclear. Transferrin is synthesised, glycosylated and secreted by the liver and the rate of transferrin synthesis is reduced in cirrhotic patients[488]. Furthermore insulin resistance is present in nearly all patients with cirrhosis[358] and thus similar mechanisms may reduce the CDT response to alcohol in the setting of cirrhosis and overweight/obesity.

In conclusion, %CDT has limited sensitivity as an objective biomarker to identify subjects consuming harmful amounts of alcohol. In our cohort of sustained heavy drinkers, diagnostic sensitivity of %CDT was 50% and yielded false negative results in particular patient subgroups: women, patients with cirrhosis and those with an elevated BMI. Therefore caution should be applied when ordering and interpreting %CDT results in these subject populations. Further studies with larger numbers of well-characterised patients, who consume heavy amounts of alcohol, are required to further assess factors which impact on the sensitivity of this assay.

CHAPTER 6

ELF SCORE ≥9.8 INDICATES ADVANCED HEPATIC FIBROSIS AND IS INFLUENCED BY AGE, STEATOSIS AND HISTOLOGICAL ACTIVITY

Fagan KJ[1,2], Pretorius C[3,4], Horsfall L[1,2], Irvine K[2], Wilgen U[3,4], Choi K[2], Fletcher L[1,3], Tate J[4], Melino M[2], Nusrat S[2], Miller G[2], Clouston A[2], Ballard E[5], O'Rourke P[5], Lampe G[6], Ungerer J[4], Powell E[1,2] *Liver International. 2015;* ***35****: 1673-1681.*

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Contributor	Statement of contribution	Contributor	Statement of contribution
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Carel Pretorius *Pathologist*	Performed ELF analysis (30%) Edited the paper (2%)	**Gregory Miller** *Histopathologist*	Performed digital image analysis (100%)
Leigh Horsfall *Clinical research coordinator*	Collected data and samples (30%)	**Andrew Clouston** *Histopathologist*	Histopathological assessment (40%) Edited the paper (2%)
Katharine Irvine *Research fellow*	Data analysis (5%) Edited the paper (10%)	**Emma Ballard** *Biostatistician*	Data analysis (20%) Edited the paper (15%)
Urs Wilgen *Scientist*	Performed ELF analysis (10%)	**Peter O'Rourke** *Biostatistician*	Data analysis (15%) Edited the paper (15%)
Kihoon Choi *Medical student*	Collected data and samples (10%)	**Guy Lampe** *Histopathologist*	Histopathological assessment (60%)
Linda Fletcher *Scientist*	Collected data and samples (5%) Edited the paper (2%)	**Jacobus Ungerer** *Pathologist*	Performed ELF analysis (30%) Edited the paper (2%)
Jill Tate *Scientist*	Performed ELF analysis (30%) Edited the paper (2%)	**Elizabeth Powell** *Principal supervisor*	Conceived and designed study (50%) Data analysis (10%) Edited the paper (40%)
Michelle Melino *Scientist*	Collected data and samples (5%)		

Abstract

Background and Aims: There is increasing need to identify individuals with advanced liver fibrosis, who are at risk of complications such as hepatocellular carcinoma. The commercially available Enhanced Liver Fibrosis (ELF) test provides a non-invasive assessment of fibrosis severity. This study was designed to determine the diagnostic accuracy of the manufacturer's cut-off value (≥9.8) in identifying advanced fibrosis.

Methods: The relationship between ELF score and fibrosis was examined using serum collected at time of liver biopsy for investigation of liver disease, particularly viral hepatitis. Fibrosis was staged using a modified METAVIR score. If available, liver tissue was recut and stained with Sirius red to determine collagen proportional area and subsinusoidal fibrosis.

Results: ELF score ≥9.8 had a sensitivity of 74.4% and specificity 92.4% for detecting advanced fibrosis. In the whole cohort (n=329), ELF score was more likely to *in*correctly classify individuals if age was ≥45 years and METAVIR inflammatory grade was 2 or 3 (adjusted OR 3.71 and 2.62 respectively). In contrast, ELF score was *less* likely to misclassify individuals in the presence of steatosis (OR 0.37). Neither subsinusoidal fibrosis nor collagen proportional area explained the discordance in ELF score for patients with or without advanced fibrosis.

Conclusion: Although ELF score ≥9.8 reliably identifies advanced fibrosis in patients with chronic liver disease, both age and inflammatory activity need to be considered when interpreting the result. Importantly, ELF score performed well in the presence of steatosis and could thus be helpful in the assessment of fatty liver disease.

6.1 Introduction

There has been a marked increase in demand for management of chronic liver disease (CLD) over the last 10 years, largely due to increasing prevalence of non-alcoholic fatty liver disease (NAFLD) and hazardous alcohol consumption, and aging of the viral hepatitis C (HCV) and B (HBV) infected cohorts. Despite progressive hepatic fibrosis, CLD has a substantial latency period during which affected individuals may lack obvious signs or symptoms of disease. Most of the morbidity and mortality associated with liver disease occurs in patients with advanced fibrosis, who are at risk of developing complications of end-stage liver disease and hepatocellular cancer. Detection and quantification of hepatic fibrosis is important within the hepatology clinic to determine prognosis, monitor disease progression and to decide if and when to administer therapy. Given the high prevalence of abnormal liver enzymes in the general (Australian) population[489] there is a need for a non-invasive test to identify people with advanced fibrosis who require specialist care.

Historically, liver biopsy has been the only reliable method to assess hepatic fibrosis, but as it is invasive and costly, it is not a suitable screening test and has limited use in monitoring disease progression. Consequently there has been substantial research to identify non-invasive methods for fibrosis assessment, including imaging techniques and serum biomarkers. Transient elastography (TE), one of the most frequently used non-invasive techniques to assess hepatic fibrosis, provides a reliable method for detecting cirrhosis and excluding significant fibrosis[239]. However, due to the need for specialized instrumentation and expertise, its use is largely limited to hepatology centres and thus in contrast to biochemical assays, it is not suitable for widespread use in general clinical practice. A number of serum tests have been developed using direct (reflecting pathophysiology of hepatic fibrogenesis) or indirect (reflecting functional alterations of the liver) biomarkers alone or in combination. Although simple panels (e.g. aspartate aminotransferase (AST) to platelet ratio index (APRI) and FIB-4) are cheaper, easier to calculate and readily available, they are not as accurate or reproducible for detecting advanced fibrosis as complex

panels, such as the commercially available Enhanced Liver Fibrosis (ELF) test[184,197].

The ELF test measures 3 direct markers of liver matrix metabolism in serum: hyaluronic acid (HA), tissue inhibitor of metalloproteinase-1 (TIMP-1) and propeptide of type III procollagen (PIIINP), which are combined to calculate the ELF score. The ELF score was validated for liver fibrosis assessment in a large group of patients with various chronic liver diseases[196] and has also been confirmed in specific patient cohorts[197-200,202,490,491]. More recent studies suggest that ELF score is influenced by age, gender and liver inflammatory activity, and that these factors may confound diagnosis at earlier fibrosis stages[201,219]. However it remains unclear whether these clinicopathological variables affect diagnostic sensitivity or specificity of the test in clinical practice, particularly for detecting advanced fibrosis.

Diagnostic accuracy of the ELF score has so far been determined by comparison with fibrosis staging based on liver biopsy[193,196]. Histological staging systems are semi-quantitative and defined by morphological changes, rather than total amount of liver fibrosis, and lack assessment of fibrous septa thickness or subsinusoidal fibrosis (SSF). In contrast, measurement of the proportion of liver biopsy occupied by collagen (collagen proportional area (CPA)) provides a quantitative assessment of liver fibrosis with less inter- and intra-observer variability[170,492]. To our knowledge, correlation of the ELF score with CPA or SSF has not been evaluated.

The main aim of this study was to evaluate the ELF test in a large group of Australian patients with CLD of mixed etiology, to determine the diagnostic accuracy of the manufacturer's cut-off value in identifying advanced fibrosis. Secondly, we investigated the influence of other clinical or histological variables on the diagnostic accuracy of the ELF test; including age, gender, disease etiology, body mass index (BMI), steatosis, SSF and inflammatory activity. The third aim was to determine the relationship between ELF score and CPA as an alternative measure of advanced fibrosis.

6.2 Materials and Methods

6.2.1 Patients and clinical data

ELF score was measured in 536 consecutive patients who underwent liver biopsy at the Princess Alexandra Hospital, Brisbane between 1999 and 2013. Informed written consent was obtained from each patient and the protocol was approved by the Metro South Health and The University of Queensland Human Research Ethics Committees. Patients were excluded if liver biopsy specimens were <15mm, unless shorter cores showed definite cirrhosis (METAVIR cohort), or <10mm length (CPA cohort), or they had stage 5 chronic kidney disease (estimated glomerular filtration rate <15), acute liver failure, drug induced liver injury, history of organ transplant, extrahepatic fibrosis, immunomodulator or antiviral therapy within 6 months, current cancer, or heavy alcohol consumption within 6 months of liver biopsy[196]. Patients were also excluded if the serum sample was grossly hemolysed, as per manufacturer instructions[223] (Figure 6.1). In the METAVIR cohort 10 patients had 2 or more liver biopsies performed (median time between biopsies 5.5 years), which were included as independent events.

Diagnosis of liver disease was based on standard biochemical and serological assays and histological assessment of the liver biopsy. Although the cohort was unselected there was a predominance of viral hepatitis due to the prior requirement to undertake a liver biopsy as a prerequisite for antiviral treatment. Weight, height, and average alcohol intake (g/day) were obtained from research nurse interview at the time of liver biopsy and alcohol intake corroborated by longitudinal review of medical records. Previous heavy alcohol use was defined as ≥350g/week for women and ≥420g/week for men, for >6 months. Information retrieved from the medical records included basic demographic details, ethnicity, previously diagnosed liver disease and other medical conditions, medications and history of tobacco and recreational drug use.

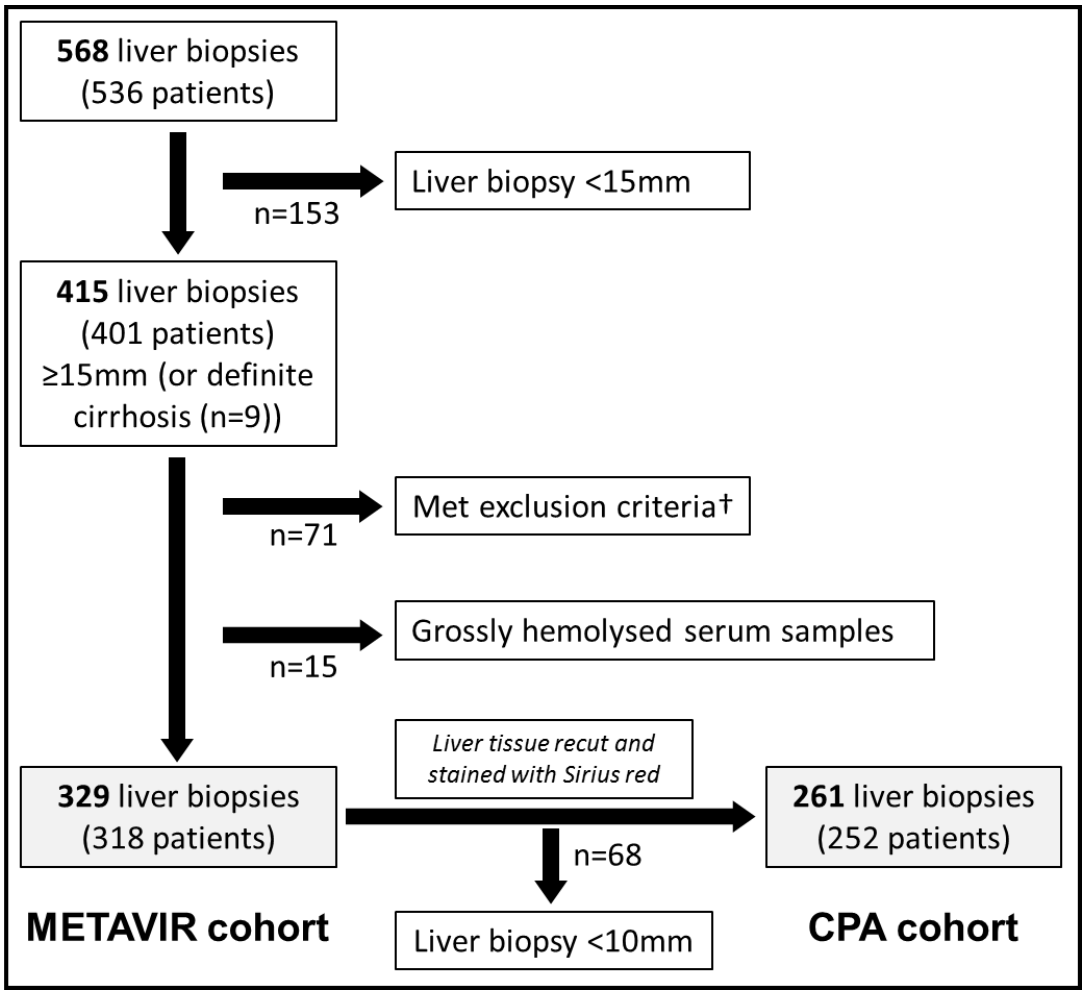

Figure 6.1. Flow chart illustrating the "METAVIR" and "CPA" cohorts and the liver biopsies excluded from analysis. †Exclusion criteria: stage 5 chronic kidney disease; acute liver failure; drug induced liver injury; organ transplant; extrahepatic fibrosis; immunomodulator or antiviral therapy; current cancer and heavy alcohol consumption.

6.2.2 Histopathological analysis

Liver biopsies were originally fixed in 10% buffered formalin, embedded in paraffin, and standard 5μm sections cut and stained with haematoxylin-eosin for histopathological assessment. The extent of fibrosis was assessed using a modified METAVIR score (modified from [163]) as follows: stage 1, portal or central fibrosis; stage 2, some septa; stage 3, many septa; stage 4, cirrhosis. Advanced fibrosis was defined as modified METAVIR fibrosis stage 3 and 4. The METAVIR scoring system was used to assess hepatic inflammatory

activity[162]. Steatosis was graded as follows: 0 (<5% parenchyma affected); 1 (5 to 33% of parenchyma affected); 2 (34 to 66% of parenchyma affected); or 3 (>66% of parenchyma affected). Additional liver tissue, if available, was recut and stained with haematoxylin-eosin and Sirius red and assessed by a single experienced hepatopathologist (GL) as above. When fibrosis stage was significantly different from the original reported fibrosis stage or resulted in a change of category from non-advanced to advanced fibrosis, the biopsy was reviewed by 2 experienced hepatopathologists (AC, GL) and a consensus stage agreed upon.

Subsinusoidal/perisinusoidal fibrosis as seen in the Sirius red-stained sections had 2 patterns. Coarse SSF, readily seen with a 4x objective in centrilobular or periseptal regions, was graded from 0-2 according to extent: 0, none; 1, mild (<50% of centrilobular regions); 2, moderate (>50% of centrilobular regions)[165]. A second pattern of very fine, non-zonal SSF, identified only at higher magnification, was scored as the percentage of sinusoids involved.

6.2.3 Quantitation of collagen proportional area (CPA)

Liver biopsies stained with Sirius red were scanned using the Aperio Scanscope XT Digital Slide Scanner (Aperio, Vista, CA, USA) at 40x magnification. The image was viewed using Aperio ImageScope software version 11.2.0.780. The liver capsule and large portal tracts (>400μm in diameter) were excluded as these do not represent disease related collagen[168]. The optimum threshold for positive pixels that corresponded to the areas of Sirius red staining was determined in 10 test cases by adjusting the hue value, colour saturation and intensity, using the original images for comparison, and the same values were subsequently used for every case (hue value 0, hue width 0.2, saturation threshold 0.22). A binary image was produced and the CPA was expressed as a percentage of positive pixels to total pixels.

6.2.4 ELF score

Serum was collected at the time of liver biopsy following an overnight fast for 8-10 hours and stored at -80ºC. Serum samples were centrifuged for 10 min at 3000g after thawing and the analyses were performed over 3 consecutive days.

An ADVIA Centaur XP system (Siemens Healthcare Diagnostics, Tarrytown, New York, USA) was used to quantify HA, PIIINP and TIMP-1 according to manufacturer's instructions. The ELF score was auto-calculated by the instrument. The manufacturer recommends a cutoff ≥9.8 for severe fibrosis[223]. The original ELF (OELF) score was calculated using the algorithm: DS = – 6.38 – (ln(age)*0.14) + (ln(HA)*0.616 + (ln(PIIINP)*0.586) + (ln(TIMP1)*0.472)[197].

6.2.5 Statistical Analysis

Statistical analyses were performed using SPSS version 22 (SPSS, Inc., Chicago, IL, USA). Categorical variables were analyzed using the Pearson Chi-Square test or Fisher's Exact test. Continuous variables were analyzed using the Student t-test or Mann-Whitney U test. Discordance between ELF score and modified METAVIR fibrosis stage was defined as ELF score ≥9.8 in individuals without advanced fibrosis or ELF score <9.8 in subjects with histological advanced fibrosis. Discordance was analysed using logistic regression with backward elimination of non-significant terms for multivariate models. A p-value of <0.05 was considered significant. Analysis was completed on the METAVIR cohort (n=329 liver biopsies) and those variables identified as significant were further examined in sub-group analyses for patients with and without advanced fibrosis. Analysis was also completed for the CPA cohort (n=261), using the same variables identified in the full model with the introduction of coarse SSF. Discordance between CPA as an alternative measure for fibrosis and ELF score, as defined above, was examined using cross-tabulation with calculation of specificity, sensitivity, positive predictive value and negative predictive value.

6.3 Results

6.3.1 Patient characteristics at liver biopsy

Five hundred and thirty-six consecutive patients had 568 liver biopsies. Paired serum and histological staging/grading were available for 415 liver biopsies (from 401 patients) with length ≥15 mm or with definite cirrhosis. Of these, 86 were excluded (including 15 grossly hemolysed serum samples), therefore the final "METAVIR cohort" consisted of 329 liver biopsies (from 318 patients)

(Figure 6.1). Demographic and clinical statistics for the comparison between the METAVIR cohort and the patients removed from analysis by the exclusion criteria are summarized in Table 6.1. Statistically significant differences were likely accounted for by the exclusion criteria and therefore the sample of 329 is representative of the population.

The 261 liver biopsies in the CPA cohort were stained with Sirius Red to enable investigations into the relationship between ELF score, SSF and CPA (Figure 6.1). It has been shown that biopsies of 10mm are sufficient for reliable CPA assessment[170]. A comparison between patients common to the METAVIR and CPA cohorts (n=261) and those only in the METAVIR cohort (n=68) showed minor differences in ethnicity (p=0.020), disease etiology (p=0.037) and modified METAVIR inflammatory grade (p=0.021) (Table 6.2). All other variables were not statistically different.

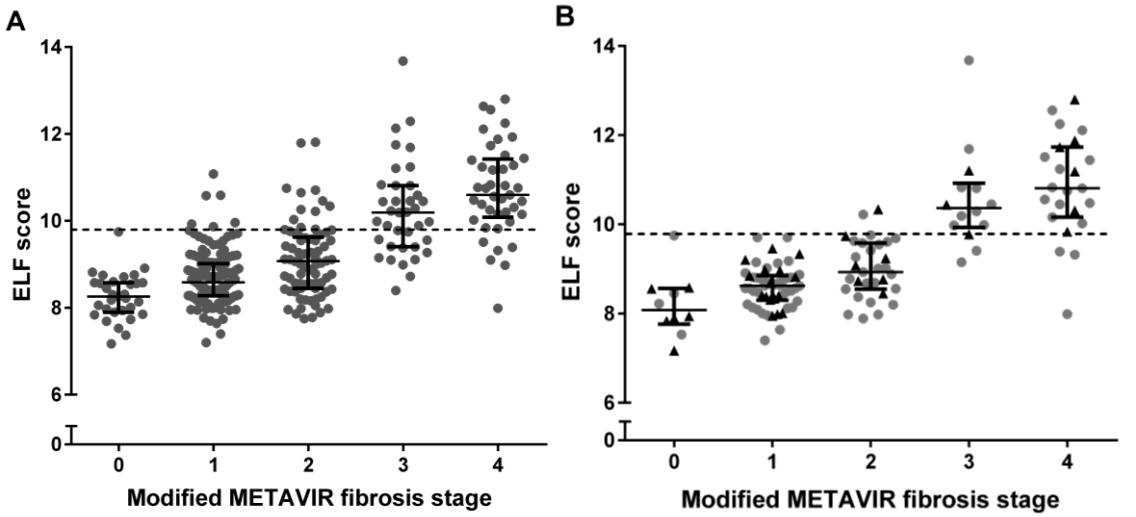

Figure 6.2. A. ELF score according to modified METAVIR fibrosis stage (METAVIR cohort). **B.** ELF score according to modified METAVIR fibrosis stage for overweight/obese patients with steatosis on liver biopsy. ▲= patients with fatty liver disease; ● = patients with all other etiologies of liver disease. The horizontal line represents ELF score 9.8.

Table 6.1. Summary of demographic and clinical statistics for the comparison between the METAVIR cohort and those patients excluded from analysis by the exclusion criteria.

Variables	METAVIR Cohort n=329	Excluded patients n=239	p-value
Age (n, % ≥45 years) †	151 (45.9)	112 (47.3)	0.749
Gender (n, % men)	211 (64.1)	153 (64.0)	0.977
Ethnicity (n, % Caucasian)	239 (72.6)	186 (77.8)	0.160
Ethnic specific Body Mass Index ‡			
Healthy (n, %)	119 (37.3)	87 (38.5)	
Overweight (n, %)	121 (37.9)	87 (38.5)	0.891
Obese (n, %)	79 (24.8)	52 (23.0)	
Disease etiology			
HBV (n, %)	63 (19.1)	40 (16.7)	
HCV (n, %)	198 (60.2)	127 (53.1)	0.019
Fatty liver (n, %)	46 (14.0)	38 (15.9)	
Other (n, %)	22 (6.7)	34 (14.2)	
Diabetes (n, %) §	34 (10.3)	25 (10.5)	0.934
Dyslipidemia (n, %) ‖	42 (12.8)	33 (13.9)	0.688
Hypertension (n, %) ¶	53 (16.1)	45 (19.0)	0.372
Smoking (pack years) ††			
None (n, %)	123 (42.0)	80 (37.4)	
1-10 years (n, %)	50 (17.1)	38 (17.8)	0.569
>10 years (n, %)	120 (41.0)	96 (44.9)	
Significant alcohol consumption at biopsy (n, %) ‡‡	21 (6.4)	27 (11.4)	0.034
Previous heavy alcohol consumption (n, %) §§	94 (28.6)	74 (31.4)	0.476
Modified METAVIR fibrosis stage Advanced (3,4)	78 (23.7)	33 (13.8)	0.003
METAVIR inflammatory grade High (2,3) ‖‖	106 (32.2)	60 (25.4)	0.080
Steatosis (n, % present)	183 (55.6)	134 (56.1)	0.916

Table 6.1 notes. †"Excluded patients" n=237. ‡"METAVIR cohort" n=319, "Excluded patients" n=226. §"Excluded patients" n=237. ‖"Excluded patients" n=237. ¶"Excluded patients" n=237. †† "METAVIR cohort" n=293, "Excluded patients" n=216. ‡‡Significant alcohol consumption defined as >140g/week women, >210g/week men; "Excluded patients" n=236. §§"Excluded patients" n=236. ‖‖"Excluded patients" n=236.

Table 6.2. Summary of demographic and clinical statistics for the METAVIR cohort and for comparison between subjects in common with the METAVIR and CPA cohorts and those subjects only in the METAVIR cohort.

Variables	METAVIR cohort (biopsy ≥15mm or definite cirrhosis) n=329	METAVIR cohort: CPA cohort (recut liver section ≥10mm) n=261	METAVIR cohort: Excluded from CPA cohort (recut liver section <10mm) n=68	p-value†
Age (n, % ≥45 years)	151 (45.9)	119 (45.6)	32 (47.1)	0.829
Gender (n, % men)	211 (64.1)	165 (63.2)	46 (67.6)	0.498
Ethnicity (n, % Caucasian)	239 (72.6)	182 (69.7)	57 (83.8)	0.020
Ethnic specific Body Mass Index ‡				
Healthy (n, %)	119 (37.3)	96 (38.1)	23 (34.3)	
Overweight (n, %)	121 (37.9)	94 (37.3)	27 (40.3)	0.844
Obese (n, %)	79 (24.8)	62 (24.6)	17 (25.4)	
Disease etiology				
HBV (n, %)	63 (19.1)	56 (21.5)	7 (10.3)	
HCV (n, %)	198 (60.2)	153 (58.6)	45 (66.2)	0.037
Fatty Liver (n, %)	46 (14.0)	32 (12.3)	14 (20.6)	
Other (n, %) §	22 (6.7)	20 (7.7)	2 (2.9)	
Diabetes (n, %)	34 (10.3)	26 (10.0)	8 (11.8)	0.664
Dyslipidemia (n, %)	42 (12.8)	33 (12.6)	9 (13.2)	0.896
Hypertension (n, %)	53 (16.1)	40 (15.3)	13 (19.1)	0.449
Smoking (pack years) ¶				
None (n, %)	123 (42.0)	96 (41.4)	27 (44.3)	
1-10 years (n, %)	50 (17.1)	42 (18.1)	8 (13.1)	0.653
>10 years (n, %)	120 (41.0)	94 (40.5)	26 (42.6)	
Significant alcohol consumption at biopsy (n, %) ††	21 (6.4)	19 (7.3)	2 (2.9)	0.269
Previous heavy alcohol consumption (n, %)	94 (28.6)	77 (29.5)	17 (25)	0.464

Modified METAVIR fibrosis stage Advanced (3,4)	78 (23.7)	57 (21.8)	21 (30.9)	0.118
METAVIR inflammatory grade High (2,3)	106 (32.2)	92 (35.2)	14 (20.6)	0.021
Steatosis (n, % present)	183 (55.6)	139 (53.3)	44 (64.7)	0.091

Table 6.2 notes. †p-value is for comparison between the "CPA cohort" and "Excluded from CPA cohort". ‡"METAVIR cohort" n=319, "CPA cohort" n=252, "Excluded from CPA cohort" n=67. §Other ("METAVIR cohort"): autoimmune liver disease (n=13) seroconverted HBV (n=1), abnormal liver function tests associated with endocrine disease (n=3), hemochromatosis (n=3), nondiseased liver (n=2). ¶"METAVIR cohort" n=293, "CPA cohort" n=232, "Excluded from CPA cohort" n=61. ††Significant alcohol consumption defined as >140g/week women, >210g/week men.

6.3.2 Diagnostic accuracy of ELF score for prediction of advanced fibrosis and validation of the manufacturer's cut-off (METAVIR cohort)

ELF score ranged from 7.2 to 13.7. Figure 6.2A shows the ELF score in patients with modified METAVIR fibrosis stages 0, 1, 2, 3 and 4. Seventy seven (23.4%) of the 329 biopsies were associated with an ELF score ≥9.8. Using a threshold ELF score of 9.8, the sensitivity of the ELF test for identifying advanced fibrosis (stage 3 or 4) was 74.4% and specificity 92.4%; the negative predictive value was 92.1% and positive predictive value was 75.3%.

In comparison to advanced fibrosis, the ELF test was less accurate as a diagnostic test for significant fibrosis (≥F2) and cirrhosis (F4) (Table 6.3). To further validate the optimal cut-off for advanced fibrosis a criterion of maximum sensitivity plus specificity was screened. Values of ELF score in the range 8.9 to 10.0 gave close to optimal cut-off points, with 9.4 being the optimal (sensitivity + specificity = 1.68), which was almost identical (1.67) to the 9.8 cut-off.

Table 6.3: Area under the receiver operating characteristic curve (AUROC) for significant, advanced and cirrhotic fibrosis and the ELF score cut-off and sensitivity to give 90% specificity for diagnosis of each category.

Fibrosis category	AUROC 95% CI	Cut-off	Sensitivity
≥ modified METAVIR F2 (significant)	0.81 (0.77-0.86)	9.5	57.1 %
≥ modified METAVIR F3 (advanced)	0.91 (0.88-0.95)	9.7	76.9 %
modified METAVIR F4 (cirrhosis)	0.90 (0.84-0.95)	10.2	68.3 %

6.3.3 Clinicopathological characteristics of patients with discordant ELF scores (METAVIR cohort)

Using an ELF score cut-off of ≥9.8 to identify advanced fibrosis, a total of 39 patients would have been incorrectly diagnosed. Nineteen of 251 patients (7.6%) with fibrosis stage 0-2 had ELF score ≥9.8, of whom 13 (68.4%) had significant (stage 2) fibrosis. False positive ELF scores were significantly associated in univariate analysis with age ≥45 ($p<0.001$), higher METAVIR inflammatory grade ($p<0.001$), elevated AST (>2x upper limit of normal (ULN), $p=0.001$), healthy BMI ($p=0.026$) and disease etiology ($p=0.020$) (Table 6.4).

Table 6.4. Demographic and clinical details of patients without advanced fibrosis and ELF score, < or ≥ 9.8

Variables	ELF Score <9.8 (n=232)	ELF Score ≥9.8 (n=19)	p-value
Age (n, % ≥45 years)	82 (35.3)	16 (84.2)	<0.001
Gender (n, % men)	137 (59.1)	14 (73.7)	0.210
Ethnicity (n, % Caucasian)	169 (72.8)	14 (73.7)	0.937
Ethnic specific Body Mass Index †			
Healthy (n, %)	83 (37.4)	13 (68.4)	
Overweight (n, %)	90 (40.5)	3 (15.8)	0.026
Obese (n, %)	49 (22.1)	3 (15.8)	
Disease etiology			
HBV (n, %)	43 (18.5)	1 (5.3)	
HCV (n, %)	141 (60.8)	12 (63.2)	0.020
Fatty liver (n, %)	33 (14.2)	1 (5.3)	
Other (n, %) ‡	15 (6.5)	5 (26.3)	
Diabetes (n, %)	16 (6.9)	1 (5.3)	1.000
Dyslipidemia (n, %)	26 (11.2)	3 (15.8)	0.468
Hypertension (n, %)	31 (13.4)	3 (15.8)	0.729
Smoking (pack years) §			
None (n, %)	89 (43.8)	8 (42.1)	
1-10 (n, %)	38 (18.7)	4 (21.1)	0.969
>10 (n, %)	76 (37.4)	7 (36.8)	
Significant alcohol consumption at biopsy (n, %) ‖	15 (6.5)	2 (10.5)	0.626
Previous heavy alcohol consumption (n, %)	64 (27.6)	6 (31.6)	0.709
METAVIR inflammatory grade 2, 3 (n, %)	50 (21.6)	12 (63.2)	<0.001
Steatosis (present) (n, %)	123 (53.0)	6 (31.6)	0.072
Alanine transaminase (n, % >2x ULN) ¶	149 (64.2)	15 (78.9)	0.195
Aspartate transaminase (n, % >2x ULN) ††	42 (18.1)	10 (52.6)	0.001
Mean cell volume (n, % ≥97 (fL))	35 (15.2)	3(15.8)	1.000
Alkaline phosphatase (n, % ≥120 (U/L))	30 (12.9)	5 (26.3)	0.157
Platelets (n, % <150 x 10^9/L)	17 (7.3)	4 (21.1)	0.061

Table 6.4 notes. † *"ELF score <9.8" n=222. ‡ ‖Other (ELF score <9.8 / ≥9.8): autoimmune liver disease (n=8 / 4), abnormal liver function tests associated with endocrine disease (n=3 / 0), hemochromatosis (n=2 / 1), non-diseased liver (n=2 / 0). §"ELF score <9.8" n=203. ‖Significant alcohol consumption defined as >140g/week women, >210g/week men. ¶Upper limit of normal (ULN) ALT = 19 (U/L) women, 30 (U/L) men. ††ULN AST = 35. (IQR = interquartile range).

Twenty of 78 patients (25.6%) with advanced fibrosis had ELF score <9.8, of whom 6 of 41 (14.6%) had cirrhosis and 14 of 37 (37.8%) had stage 3 fibrosis. False negative ELF scores were associated in univariate analysis with absence of steatosis (p=0.006), lower ALT (<2x ULN, p=0.012), and disease etiology (p=0.033) (Table 6.5).

In order to identify the clinicopathological variables independently associated with discordant ELF scores in the whole cohort, multivariate logistic regression analysis was performed. Overall, ELF discordance was best explained by age, METAVIR inflammatory grade and steatosis. ELF score was more likely to *in*correctly classify individuals if age was ≥45 years and METAVIR inflammatory grade was 2 or 3 (adjusted odds ratio (OR) 3.71 (95% CI 1.74-7.89) and 2.62 (95% CI 1.3-5.3) respectively). In contrast, the ELF score was *less* likely to *in*correctly classify individuals in the presence of steatosis (OR 0.37 (95% CI 0.18-0.75)) (Table 6.6).

These 3 variables were further examined in sub-group analyses for patients with and without advanced fibrosis (Table 6.6). Age ≥45 years and METAVIR inflammatory grade (2/3) were positively associated with discordant ELF score results for non-advanced fibrosis (false positive) and negatively associated for advanced fibrosis (false negative). In contrast the presence of steatosis was associated with fewer discordant ELF score results for patients with *and* without advanced fibrosis.

In view of the influence of age on the ELF score, the data was compared to the original ELF score, which included age in the algorithm. Both scores were significantly correlated (r=1.0, p<0.001).

Table 6.5. Demographic and clinical details of patients with advanced fibrosis and ELF score, < or ≥ 9.8

Variables	ELF Score <9.8 (n=20)	ELF Score ≥9.8 (n=58)	p-value
Age (n, % ≥45 years)	12 (60.0)	41 (70.7)	0.377
Gender (n, % men)	17 (85.0)	43 (74.1)	0.376
Ethnicity (n, % Caucasian)	14 (70.0)	42 (72.4)	0.836
Ethnic specific Body Mass Index			
Healthy (n, %)	7 (35.0)	16 (27.6)	
Overweight (n, %)	7 (35.0)	21 (36.2)	0.799
Obese (n, %)	6 (30.0)	21 (36.2)	
Disease etiology			
HBV (n, %)	9 (45.0)	10 (17.2)	
HCV (n, %)	9 (45.0)	36 (62.1)	0.033
Fatty liver (n, %)	1 (5.0)	11 (19.0)	
Other (n, %) [§]	1 (5.0)	1 (1.7)	
Diabetes (n, %)	4 (20.0)	13 (22.4)	1.000
Dyslipidemia (n, %)	2 (10.0)	11 (19.0)	0.496
Hypertension (n, %)	3 (15.0)	16 (27.6)	0.369
Smoking (pack years) [*]			
None (n, %)	10 (52.6)	16 (30.8)	
1-10 (n, %)	1 (5.3)	7 (13.5)	0.208
>10 (n, %)	8 (42.1)	29 (55.8)	
Significant alcohol consumption at biopsy (n, %)[‖]	0 (0.0)	4 (6.9)	0.567
Previous heavy alcohol consumption (n, %)	6 (30.0)	18 (31.0)	0.931
METAVIR inflammatory grade 2, 3 (n, %)	8 (40.0)	36 (62.1)	0.086
Steatosis (present) (n, %)	9 (45.0)	45 (77.6)	0.006
Alanine transaminase (n, % >2x ULN) [†]	11 (55.0)	49 (84.5)	0.012
Aspartate transaminase (n, % >2x ULN) [‡]	7 (35.0)	33 (56.9)	0.091
Mean cell volume (n, % ≥97 (fL))	4 (20.0)	22 (37.9)	0.142
Alkaline phosphatase (n, % ≥120 (U/L))	3 (15.0)	15 (25.9)	0.376
Platelets (n, % <150 x 10^9/L)	9 (45.0)	31 (53.4)	0.515

Table 6.5 notes. [*]"ELF score <9.8" n=19; "ELF score ≥9.8" n=52. [‖]Significant alcohol consumption defined as >140g/week women, >210g/week men. [†]Upper limit of normal (ULN) ALT = 19 (U/L) women, 30 (U/L) men. [‡]ULN AST = 35. (IQR = interquartile range). [§]Other: "ELF score <9.8", autoimmune liver disease (n=1); "ELF score ≥9.8", seroconverted HBV (n=1).

Table 6.6. Summary of the multivariate models examining discordance† between ELF score and modified METAVIR fibrosis stage: Model for overall discordance (n=329); subgroup analysis for subjects without advanced fibrosis (n=251); subgroup analysis for subjects with advanced fibrosis (n=78)

Model	p-value	Odds ratio (95% CI)
Model for overall discordance		
Age (≥45)	0.001	3.706 (1.740-7.892)
Steatosis (present)	0.006	0.367 (0.179-0.754)
METAVIR inflammatory grade (2,3)	0.007	2.624 (1.295-5.318)
Model for non-advanced fibrosis		
Age (≥45)	<0.001	11.480 (3.090-42.651)
Steatosis (present)	0.087	0.385 (0.129-1.149)
METAVIR inflammatory grade (2,3)	<0.001	7.030 (2.435-20.296)
Model for advanced fibrosis		
Age (≥45)	0.391	0.606 (0.193-1.904)
Steatosis (present)	0.023	0.276 (0.092-0.835)
METAVIR inflammatory grade (2,3)	0.181	0.468 (0.154-1.423)

Table 6.6 notes. †Discordance between ELF score and METAVIR fibrosis stage was defined as ELF score ≥9.8 in individuals without advanced fibrosis or ELF score <9.8 in subjects with histological advanced fibrosis.

6.3.4 Influence of obesity-related steatosis on the ELF score

Obesity-related steatosis is a common cofactor in patients with other chronic liver diseases and is associated with more rapid progression of fibrosis[493-495]. Although only 46 patients had a primary diagnosis of fatty liver (39 of whom had NAFLD), obesity-related steatosis was a common cofactor in the cohort; 132 (40%) of liver biopsies had evidence of steatosis in the setting of an elevated BMI. Figure 6.2B illustrates the ELF score according to fibrosis stage for overweight/obese patients with steatosis on liver biopsy. For this subgroup of patients the sensitivity of ELF score ≥9.8 to identify advanced fibrosis was 83.9% and specificity 97.9%; the negative predictive value was 93.9% and the positive predictive value was 93.9%.

6.3.5 Relationship between ELF score and SSF (CPA cohort)

The pattern and extent of collagen deposition was assessed in the 261 liver biopsies in the CPA cohort that were stained with Sirius red. As well as septal fibrosis, collagen deposition in the subsinusoidal space of Disse was present and was composed either of conspicuous and readily identifiable collagen bundles (coarse SSF) or very fine and subtle collagen strands (fine SSF). Coarse SSF, observable at low power magnification in a pattern similar to that seen in steatohepatitis, was seen in 146 (55.9%) liver biopsies of the CPA cohort. Twenty-three of the 32 (71.9%) patients with fatty liver disease (NAFLD and alcohol related liver disease) had higher grades of coarse SSF compared with HCV (17.6%), HBV (19.6%) or other disease etiology (20.0%) ($p<0.001$). Conditions traditionally associated with SSF (steatosis ($p=<0.001$), type 2 diabetes ($p<0.001$), dyslipidemia ($p<0.001$), hypertension ($p=0.013$)) and METAVIR inflammatory grade ($p<0.001$) were significantly associated with higher grade of coarse SSF. Overall, the extent of coarse SSF was positively associated with modified METAVIR fibrosis stage ($p<0.001$) and with ELF score ($p<0.001$). However, addition of coarse SSF to the logistic regression model, using data from the 261 patients in the CPA cohort, demonstrated that the presence or extent of coarse SSF did not explain discordant ELF scores ($p=0.12$).

In contrast to coarse SSF, very fine, non-zonal SSF was detected in all liver biopsies on high power analysis of Sirius red-stained sections. It was typically more delicate and widespread compared with SSF seen in association with steatohepatitis (Figure 6.3) and involved 5% to 95% of sinusoids. Conditions traditionally associated with coarse SSF were not significantly associated with the extent of fine SSF (data not shown). In contrast to coarse SSF, fine SSF was not associated with modified METAVIR fibrosis stage ($p=0.13$) or the ELF score ($p=0.77$).

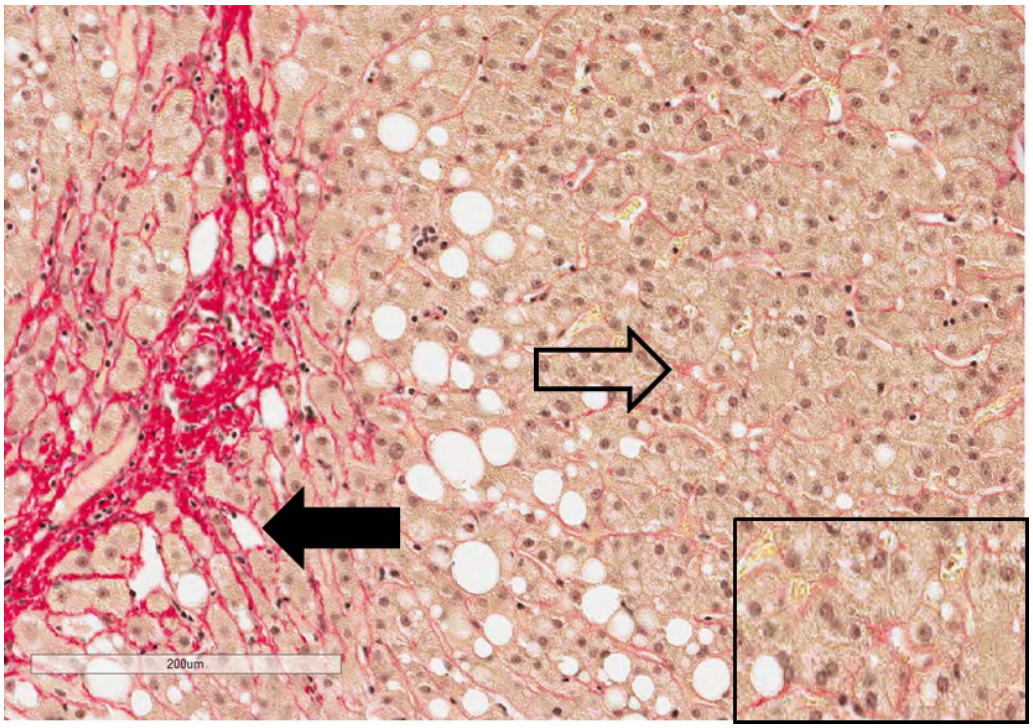

Figure 6.3. Fibrosis in steatohepatitis. Coarse subsinusoidal and pericellular fibrosis is present adjacent to a fibrous septa (solid arrow). Very fine and diffuse collagen deposition is also present throughout the remaining parenchyma (open arrow and inset).

6.3.6 Relationship between ELF score and collagen proportional area (CPA cohort)

Hepatic CPA ranged from 0.4 to 35.5%. Although a significant relationship was seen between CPA and modified METAVIR fibrosis stage ($p<0.001$) there was substantial overlap between individual fibrosis stages. Overall, CPA correlated with ELF score ($r_s=0.34$, $p=<0.001$), but there was no significant correlation *within* each stage of fibrosis (F0, p=0.78; F1, p=0.68; F2, p=0.62; F3, p=1.00; F4, p=0.53). CPA values were categorized into 4 stages: C1, 0-5%; C2, >5-10%; C3, >10-20%; C4 >20%, with stage 3 and 4 representing advanced fibrosis[169]. Discordance in ELF score with a cut-off value of 9.8 was not improved by using CPA stage as an alternative outcome measure of advanced fibrosis (sensitivity 65.0%, specificity 84.2%). As expected, CPA correlated with fine SSF overall ($r_s=0.35$, $p=<0.001$) and within each fibrosis stage except stage 0 (data not shown).

6.4 Discussion

Given the high prevalence of abnormal liver enzymes in the general Australian population[489] there is a need for a non-invasive test to identify subjects with advanced fibrosis who require specialist care. This study was undertaken to assess the diagnostic performance of the serum ELF test for identification of advanced fibrosis. In a large group of Australian patients with CLD of mixed etiology, an ELF score ≥9.8 correctly identified 74.4% of patients with advanced fibrosis and correctly excluded 92.4% of patients *without* advanced fibrosis.

The present study demonstrates that in a patient with CLD, an ELF score ≥9.8 reliably indicates the presence of advanced fibrosis. The majority of the 7.6% of patients with “false positive” ELF scores had significant (stage 2) fibrosis along with increased age and higher inflammatory scores. It is possible that these liver biopsies were misclassified due to sampling variability leading to under staging of the extent of fibrosis, or reflect the semi-quantitative nature of histological staging systems. Alternatively, ELF score ≥9.8 in these patients with less severe fibrosis may reflect active fibrogenesis, matrix turnover and liver inflammation. Both increased age and higher inflammatory scores are risk factors for progressive liver disease. Interestingly, in a study of 457 patients followed for a median of 7 years, ELF score predicted liver-related outcomes independently of liver biopsy[221]. In that study, a unit change in ELF was associated with a doubling of risk of a liver related outcome[221]. Therefore ELF score ≥9.8 in patients with less severe fibrosis may predict future liver disease progression and warrant more intensive patient review or intervention. Longitudinal clinical outcome data were not available for our current study, but are clearly important data to collect in future studies in order to interpret ELF score in clinical practice.

The 20 patients with advanced fibrosis with ELF score <9.8 are of concern, and demonstrate that a low ELF score does not exclude severe fibrosis. In our study, patients with advanced fibrosis and steatosis were less likely to be *in*correctly classified by the ELF score and this may support a role for steatosis in active fibrogenesis and matrix turnover or inflammation. The ELF test may be particularly useful as a noninvasive test for fibrosis in patients with NAFLD,

when there may be technical limitations to the use of liver stiffness measurements[247]. In contrast to fatty liver disease, significantly more patients with advanced fibrosis and hepatitis B had “false negative” ELF scores, particularly in those patients with stage 3 fibrosis and less inflammation, consistent with findings in other studies[496]. Compared to transient elastography (TE), lower performance of the ELF score in detecting advanced fibrosis has previously been reported for patients with hepatitis B[202,260,496]. Therefore in clinical practice, perhaps a lower cut-off value for ELF score is required to detect advanced fibrosis in patients with hepatitis B, and results in the setting of ALT flares interpreted with caution, similar to analysis of TE.

The relationship between ELF score and age is not unexpected. Increased age is considered to be a low grade inflammatory state[497] and is associated with fibrosis progression[498], perhaps due to increased vulnerability to environmental factors, particularly oxidative stress[499]. Extrahepatic chronic inflammatory disorders, including cardiovascular disease, are also more common in aged populations. Age was a component of the original ELF panel, but this was subsequently simplified by removing age following reports that diagnostic accuracy was not affected[199]. Our results demonstrate that increased age (≥45 years) contributes to “false positive” ELF scores in patients without advanced fibrosis and may suggest that not enough emphasis was placed on age in the original ELF algorithm.

Sirius red staining identified 2 patterns of SSF, coarse SSF, associated with fatty liver disease and metabolic risk factors, and an unusual pattern of fine diffuse SSF that was more delicate and widespread than the former. This fine SSF has infrequently been reported[160] but is likely under recognized since it is not easily seen except on high power analysis following a Sirius red stain. The lack of correlation with other markers of disease progression suggests that it is probably not a progressive lesion. Although the extent of coarse SSF was positively associated with ELF score, it did not explain the discordant ELF scores in patients.

SSF does, however, contribute to collagen content in the liver biopsy, and hence the variability in collagen proportional area between patients within each

stage of fibrosis. Although CPA provided a continuous variable for measurement of hepatic fibrosis, the results did not explain discordant ELF score results in patients with or without advanced fibrosis. Furthermore, discordance in ELF score was not improved by using CPA stage as the outcome measure of advanced fibrosis. Similar findings were seen in a study of 386 liver biopsies from patients with chronic hepatitis C[167]. In the latter study, serum fibrosis markers (TIMP-1, PIIINP, HA and YKL-40) correlated better with Ishak fibrosis score than with the log hepatic collagen content, suggesting that these markers reflect the *pattern* of fibrosis and ongoing fibrogenesis more closely than the amount of hepatic collagen[167].

Like most studies investigating non-invasive markers of hepatic fibrosis our study was performed in the context of a hepatology clinic, where patients have known, or are being investigated for liver disease. However, due to the rising prevalence of NAFLD, there is increasing demand for a simple test in primary care that can identify patients with advanced fibrosis who require referral to specialist care. Serum biomarkers are therefore suited to this role and offer considerable advantage over TE which is more expensive, requires specific expertise and may not provide valid results in obesity[247]. The ELF test performs well in the presence of steatosis and increased BMI thus it may be a practical screening test for advanced fibrosis in patients with NAFLD. Clearly our cohort suffers selection bias due to the requirement for liver biopsy and high prevalence of viral hepatitis. In addition, the prevalence of significant fibrosis (METAVIR fibrosis stage 2-4), advanced fibrosis and cirrhosis was 46.8%, 23.7% and 12.5% respectively, which is considerably higher than expected in the community[178,500]. Therefore although our study supports the potential role of ELF test as a screening tool for advanced fibrosis, further studies are required to assess its utility in a primary care cohort.

CHAPTER 7

THE BURDEN OF DECOMPENSATED CIRRHOSIS AND ASCITES ON HOSPITAL SERVICES IN A TERTIARY CARE FACILITY: TIME FOR CHANGE?

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Abstract

Background: Ascites, the most frequent complication of cirrhosis, is associated with poor prognosis and reduced quality of life. Recurrent hospital admissions are common and often unplanned, resulting in increased use of hospital services.

Aims: To examine use of hospital services by patients with cirrhosis and ascites requiring paracentesis and to investigate factors associated with early unplanned readmission.

Methods: A retrospective review of the medical chart and clinical databases was performed for patients who underwent paracentesis between October 2011 and October 2012. Clinical parameters at index admission were compared between patients with and without early unplanned hospital readmissions.

Results: The 41 patients requiring paracentesis had 127 hospital admissions, 1164 occupied bed days and 733 medical imaging services. Most admissions (80.3%) were for management of ascites, of which 41.2% were unplanned. Of those eligible, 69.7% were readmitted and 42.4% had an early unplanned readmission. Twelve patients died and 9 developed SBP. Of those eligible for readmission, more patients died ($p=0.008$) and/or developed SBP ($p=0.027$) if they had an early unplanned readmission during the study period. Markers of liver disease, as well as haemoglobin ($p=0.029$), haematocrit ($p=0.024$) and previous heavy alcohol use ($p=0.021$) at index admission, were associated with early unplanned readmission.

Conclusion: Patients with cirrhosis and ascites comprise a small population who account for substantial use of hospital services. Markers of disease severity may identify patients at increased risk of early readmission. Alternative models of care should be considered to reduce unplanned hospital admissions, health care costs and pressure on emergency services.

7.1 Introduction

The burden of liver disease is rising, due in part to increasing prevalence of non-alcoholic fatty liver disease (NAFLD), hazardous alcohol consumption, and chronic viral hepatitis B (HBV) and C (HCV). In Australia, liver disease including fatty liver affects more than a quarter of the population and in 2012 the health costs of treating liver disease were estimated to be $432 million[1]. Regardless of etiology, most of the morbidity and mortality from chronic liver disease (CLD) occurs among people with advanced fibrosis or cirrhosis, who are at risk of developing complications of cirrhosis including ascites, hepatic encephalopathy and variceal haemorrhage.

The morbidity and health care costs associated with these complications of cirrhosis are substantial. In the US, cirrhosis is responsible for more than 150,000 hospitalisations, costing in excess of US$4 billion annually[274]. Recurrent hospital admissions among this patient population are common and are associated with higher risk of subsequent mortality. A recent study from a US academic liver transplant centre found that 37% of patients with decompensated cirrhosis were readmitted within a month of discharge at a cost of over US$20,000 per admission[387]. Risk factors for readmission included liver disease severity and complexity of medical management. Importantly, 22% of hospital readmissions were judged to be possibly preventable, due to failure to appropriately titrate or monitor medications, or to plan ahead for paracentesis.

Ascites is the most frequent complication of cirrhosis and is associated with poor prognosis, reduced quality of life and increased hospital admissions[278,402,501]. A significant complication of ascites is spontaneous bacterial peritonitis (SBP), which occurs in approximately 1.5-3.5% of outpatients and 10% of inpatients[502] and is the most common infection in patients with decompensated cirrhosis[379]. Published guidelines[373,503] and quality indicators[504] describe effective acute interventions for management of patients hospitalised with ascites. However after hospital discharge, patients receive episodic outpatient care and risk subsequent complications including reaccumulation of ascites, fluid or electrolyte imbalance and renal impairment, which may result in readmission. In other common chronic diseases such as

congestive heart failure and chronic obstructive pulmonary disease, risk factors for early readmission have been identified and institution of chronic disease management has led to a reduction in disease-related admissions and cost-savings[505,506]. In contrast, little information is available regarding factors that predict hospital readmission in Australian patients with ascites.

The main aim of this study was to investigate the use of hospital services at a single tertiary hepatology centre over a 12 month period by patients requiring paracentesis for ascites due to decompensated cirrhosis. The second aim was to determine clinical parameters that may help identify and coordinate care for patients with early unplanned readmissions and higher care needs.

7.2 Methods

7.2.1 Patients and clinical data

A retrospective cohort investigation was conducted at the Princess Alexandra Hospital, a tertiary care facility containing a dedicated Gastroenterology and Hepatology department and the referral centre for the state-wide liver transplant service. The study protocol was approved by Metro-South Hospital and Health Services Human Research Ethics Committee. Informed consent was waived as the study data was anonymised and involved no risk to patients' rights or welfare.

Patients with CLD who underwent abdominal paracentesis at the Princess Alexandra Hospital between October 2011 and October 2012 were included in the study. Patients were identified if an ascitic fluid sample related to cirrhosis was recorded on the Queensland Pathology database during the study period. Further paracenteses were identified for these patients on review of their medical record. The first hospital admission and first paracentesis performed during the 12 month period are referred to as the "index admission" and "index paracentesis" respectively. Admissions or paracenteses were defined as "planned" if they were arranged admissions and "unplanned" if they were not scheduled. Early unplanned readmissions were defined as unplanned

readmissions that occurred within 1 month of a previous admission. Deaths were identified from the medical record and hospital based corporate information system.

Patient medical records were reviewed to obtain for each paracentesis: demographic details, previously diagnosed liver disease and other medical conditions, medications and history of tobacco and alcohol use. Current alcohol use was stratified according to whether the patient consumed less than or greater than recommended weekly allowance (RWA) of alcohol (140g/week for women, 210g/week for men), which is the threshold when liver injury is likely to occur (based on epidemiological data)[9-12]. Previous heavy alcohol use was defined as ≥350g/week for women and ≥420g/week for men, for >6 months. Results from ascitic fluid analysis and routine haematological and biochemical tests performed at each paracentesis were recorded. SBP was defined as an ascitic fluid polymorphonuclear count >250/mm^3. Standard biochemical and serological assays, liver imaging and histological assessment of a liver biopsy (if performed) were used to confirm diagnosis of liver disease and cirrhosis. The severity of liver disease was evaluated using the Child-Turcotte-Pugh (CTP) classification[382], Model for End-Stage Liver Disease (MELD) score[383] and the United Kingdom Model for End-Stage Liver Disease score (UKELD)[507]. Comorbidity was graded using the Charlson comorbidity index[508] and cirrhosis-specific comorbidity scoring system (CirCom)[509].

The outpatients scheduling information management system and the hospital radiology database were searched to identify use of outpatient and radiology services during the 12 month period. Endoscopic reports were obtained from the hospital endoscopy database for all procedures completed during the year.

7.2.2 Statistical analysis

Conventional descriptive statistics were used to describe the demographic and clinical characteristics for the whole cohort. The Mann-Whitney U test was used to test for significant differences in MELD and CTP score between those who had undergone paracenteses prior to commencement of the study and patients who experienced their first paracentesis during the study period. Survival analysis of time to readmission was completed using the Kaplan-Meier method with the event being readmission.

The second aim was to examine potential variation between those patients with and without an early unplanned readmission. Continuous variables that were not normally distributed or had heterogeneity were examined using the Mann-Whitney U test. Categorical variables were examined using the Fisher's Exact test. The per month rate for the total number of paracenteses, number of admissions and the total length of hospital stay were calculated for each patient by adjusting for the period of time during the study period that the patient could potentially be readmitted following the index admission. Patients were not eligible for readmission once they died or had a liver transplant.

7.3 Results

7.3.1 Patient characteristics at index paracentesis

A total of 41 individual patients with portal hypertension and ascites requiring paracentesis were admitted over the 12 month period. The demographic and clinical data for these patients are displayed in Table 7.1. The primary cause of portal hypertension was alcohol-related liver disease in 18 patients, chronic HCV in 13, chronic HBV and hepatocellular cancer in 1, and other in 9. Previous harmful alcohol consumption was also a co-factor in 6 of the 13 patients with chronic HCV. The median MELD score was 17 (IQR: 13-21) and the median CTP score was 10 (IQR: 9-12). There was no difference in median MELD ($p=0.77$) or CTP ($p=0.48$) score between the 19 patients who had undergone paracenteses prior to commencement of the study and the 22 patients who experienced their first paracentesis during the study period.

Table 7.1. Demographic data and comorbidities at index admission for the overall cohort and for "No early unplanned readmission" and "Early unplanned readmission".

Clinical parameter	Total (n=41)	"No early unplanned readmission" (n=19)	"Early unplanned readmission" (n=14)	p-value†
Age (years) mean (±SD)	53.6 (11.8)	53.8 (13.0)	56.8 (9.7)	0.47
Gender (n, % men)	32 (78.0)	13 (68.4)	13 (92.9)	0.20
Caucasian (n, %)	34 (82.9)	15 (78.9)	11 (78.6)	1.00
Etiology of cirrhosis (n, %)				
Alcohol	18 (43.9)	6 (31.6)	8 (57.1)	0.21
Viral hepatitis	14 (34.1)	7 (36.8)	5 (35.7)	
Other‡	9 (22.0)	6 (31.6)	1 (7.1)	
Diabetes (n, %)	14 (34.1)	4 (21.1)	8 (57.1)	0.066
Metabolic risk factors§ (n, %)	17 (41.4)	8 (42.1)	6 (42.9)	1.00
Chronic airways disease (n, %)	6 (14.6)	3 (15.8)	1 (7.1)	0.62
Current alcohol consumption >RWA (n, %)	7 (17.1)	1 (5.3)	5 (35.7)	0.062
Previous heavy alcohol (n, %)	28 (68.3)	10 (52.6)	13 (92.9)	0.021
Depression (n, %)	18 (43.9)	7 (36.8)	8 (57.1)	0.30
Gastoesophageal varices (n, %)	26 (63.4)	11 (57.9)	11 (78.6)	0.28
Hepatic encephalopathy (n, %)	20 (48.8)	6 (31.6)	10 (71.4)	0.037
Charlson comorbidity index median (IQR)	4.0 (3.0-4.0)	3.0 (3.0-4.0)	4.0 (3.0-4.3)	0.36
CirCom median (IQR)	0.6 (0.0-1.0)	0.0 (0.0-1.0)	0.0 (0.0-0.5)	0.76

Table 7.1 notes. †p-value is for comparison between "No early unplanned readmission" and "Early unplanned readmissions". ‡Other: NAFLD (n=3), cystic fibrosis and biliary cirrhosis (n=1), biliary atresia (n=1), cryptogenic cirrhosis (n=1), autoimmune hepatitis (n=1), cholangiocarcinoma (n=1), congenital hepatoportal arteriovenous fistula (n=1). §Metabolic risk factors: hypertension, ischaemic heart disease, dyslipidaemia.

Medical co-morbidities were also present in this patient cohort as detailed in Table 7.1. All patients were taking medications at index presentation, with a median number of medications per patient of 6 (IQR: 2-8). Furthermore, prescriptions for medications to manage complications of cirrhosis (e.g. propranolol, lactulose and diuretics) increased over the study period, as did the use of proton pump inhibitors. Diuretic therapy was eventually prescribed to 36 (87.8%) patients during the study period, but importantly was ceased at least once for 13 patients due to acute kidney injury (n=11) and/or hyponatraemia (n=9).

7.3.2 Hospital admissions and occupied bed days

During the 12 month study period the 41 patients had a total of 127 hospital admissions. One hundred and two (80.3%) of these admissions were for the management of ascites, of which 60 (58.8%) were planned and 42 (41.2%) unplanned.

Overall there were a total of 1164 occupied bed days comprising 41 outpatient days (day procedure unit) and 1123 inpatient days. Of the inpatient days 832 (74%) were attributed to admissions for management of ascites. Median (IQR) length of inpatient stay for admissions for management of ascites was 6 (3-11) days, compared to 11 (5-27) days for admissions for another reason (e.g. gastrointestinal bleed, infection, falls). Seven patients died and one patient had a liver transplant during their index admission, thus were not eligible for readmission. Twenty-three (69.7%) patients were readmitted during the study period of which 14 (42.4%) had unplanned readmissions within a month of discharge. The median (95% CI) time to readmission for the 33 patients eligible for readmission was 68 (5.9-130.1) days following discharge from the index admission, as demonstrated in Figure 7.1. Patients were censored if they died or received a liver transplant, since this affected their likelihood of readmission. The probability (95% CI) of readmission at 1 month was 0.4 (0.2-0.6) and at 3 months, when patients with decompensated liver disease are usually scheduled for review in outpatient clinic, was 0.6 (0.4-0.8).

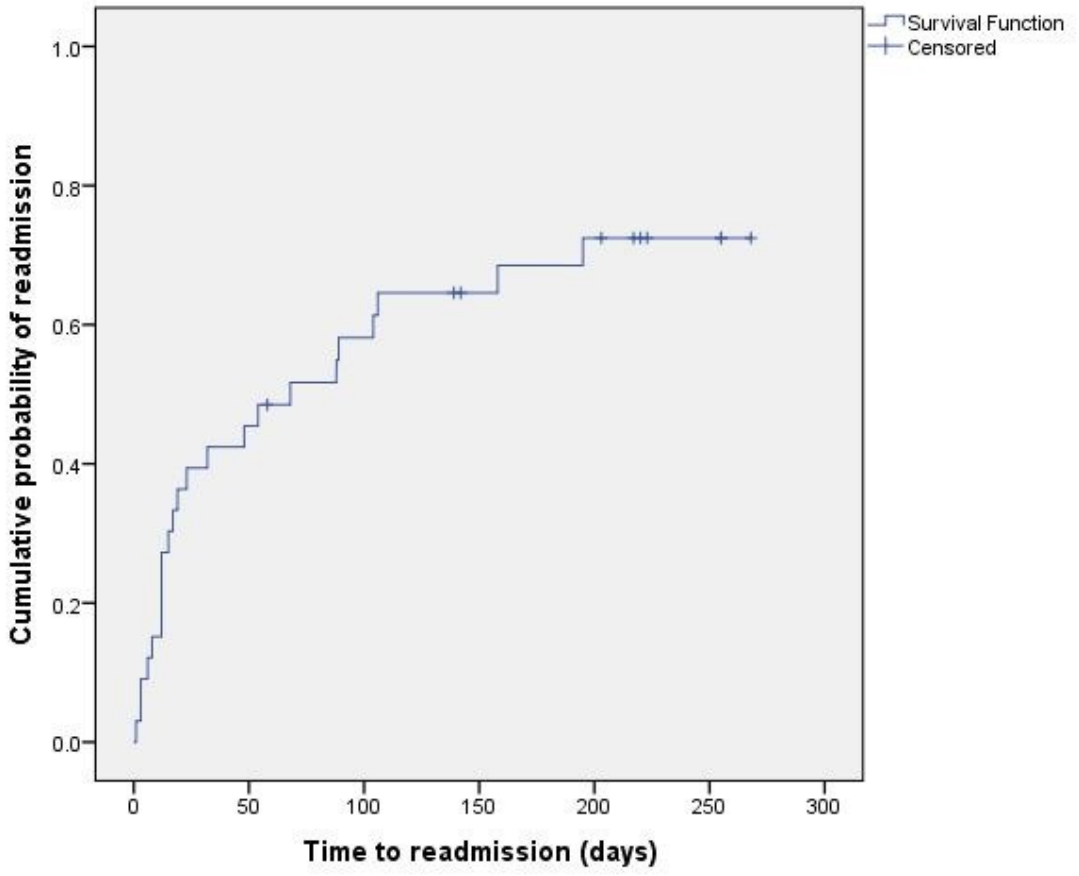

Figure 7.1. Kaplan-Meier curve for time to readmission during the study period. (Vertical line indicates censored: patient died or received liver transplant).

7.3.3 Outpatient care and medical imaging use

During the study period the patients had a total of 328 outpatient appointments: 274 (83.5%) were related to liver disease (e.g. appointments scheduled with hepatology, hepatobiliary surgery, liver transplant clinic, liver dietician). Furthermore, there were 343 “chart reviews” (patient care related events without the patient present) (e.g. to follow up laboratory tests and medical imaging and to advise about medication dosages), although the majority (98.8%) of these were for patients who had received or were being assessed for a liver transplant. Twenty-six patients had 34 endoscopic procedures performed during the study period.

The patients received 733 medical imaging services over the 12 month period: abdominal ultrasounds (n=180); CT scan (n=67); MRI (n=8), radiographs (n=418); bone mineral densitometry (n=11), and other radiological services (e.g. interventional procedures or other ultrasounds) (n=49). Five hundred and thirteen (70.0%) of these were performed on patients who presented with an unplanned admission.

7.3.4 Paracenteses

The 41 patients received a total of 206 paracenteses (median 4, IQR: 2-9). During unplanned admissions, only 25.0% of initial paracenteses occurred in a liver-related ward, with 38.3% occurring in the Emergency Department. In contrast during planned admissions, 97% of paracenteses were performed in a liver-related ward, with none occurring in the Emergency Department.

Greater than half the paracenteses (n=124, 60.2%) were performed for therapeutic purposes, with a mean (SD) volume of 7.5L (±4.0L) fluid removed during each procedure. Eighty-two (39.8%) paracenteses were performed for diagnostic purposes, usually to exclude the presence or monitor treatment of spontaneous bacterial peritonitis (SBP). Paracentesis occurred within 24 hours for 84.3% (n=107) of admissions, and in 90.3% (n=186) of procedures an ascitic fluid cell count and differential was performed. During the 12 month period SBP was confirmed in 22 paracenteses for 9 patients during 10 hospital admissions. For all of the cases, antibiotics were commenced within 24 hours of SBP diagnosis. However, 9 of the 10 admissions had a negative ascitic fluid culture on initial paracentesis, 6 of which had antimicrobials detected in the ascitic fluid.

7.3.5 Factors associated with early unplanned readmission

The second aim was addressed by comparing clinical parameters at the index paracentesis for 33 patients with (n=14) or without (n=19) early unplanned readmissions (8 patients excluded due to death (n=7) or liver transplantation (n=1) during the index admission). Comparisons between the “No early unplanned readmission” and “Early unplanned readmission” groups are shown for: demographic data and comorbidities (Table 7.1); laboratory studies and medications at index admission (Table 7.2); and care needs (Table 7.3).

Table 7.2. Laboratory studies, severity of liver disease scores and medication use at index admission for "No early unplanned readmission" and "Early unplanned readmission".

Clinical parameter	"No early unplanned readmission" (n=19)	"Early unplanned readmission" (n=14)	p-value
Serum sodium (mmol/L) median (IQR)	136.0 (131.0-139.0)	133.0 (128.0-135.5)	0.091
Serum urea (mmol/L) median (IQR)	4.7 (3.1-7.2)	6.9 (3.7-9.9)	0.23
Serum eGFR (ml/min) median (IQR)†	90.0 (69.8-90.0)	79.5 (49.0-90.0)	0.25
Serum bilirubin umol/L median (IQR)†	37.5 (18.8-71.5)	51.5 (28.5-76.3)	0.21
Haemoglobin (g/L) median (IQR)	125.0 (102.0-134.0)	103.0 (89.3-120.3)	0.029
Haematocrit median (IQR)§	0.38 (0.31-0.40)	0.31 (0.26-0.36)	0.024
Mean Cell Volume (fL) median (IQR)§	96.0 (89.0-101.0)	100.0 (93.5-106.0)	0.24
C-Reactive Protein (mg/L) median (IQR)‡	13.0 (6.5-30.0)	6.1 (4.8-28.5)	0.30
Ascitic fluid total protein (g/L) median (IQR)†	14.5 (5.0-18.8)	12.0 (5.0-20.8)	0.61
CTP Score†	9.0 (8.0-11.0)	10.5 (9.0-12.0)	0.16
MELD Score†	14.5 (10.8-18.3)	17.5 (15.0-24.3)	0.030
UKELD Score†	56.0 (50.5-58.3)	57.4 (54.0-65.3)	0.091
Number of medications median (IQR)	4.0 (2.0-6.0)	6.0 (3.8-7.5)	0.23
Diuretic use (n, %)	7 (36.8)	8 (57.1)	0.30
Proton Pump Inhibitor use (n, %)	8 (42.1)	8 (57.1)	0.49
Propranolol use (n, %)	1 (5.3)	5 (35.7)	0.062
SBP prophylaxis (n, %)	1 (5.3)	2 (14.3)	0.56

Table 7.2 notes. †"No early unplanned readmission" n=18, ‡" No early unplanned readmission" n=17 and "Early unplanned readmission" n=13, §"Early unplanned readmission" n=13.

Haemoglobin (p=0.029), haematocrit (p=0.024), MELD score (p=0.030), previous heavy alcohol (p=0.021) and hepatic encephalopathy (p=0.037) were significantly different between the 2 groups. Furthermore, 5 (35.7%) patients who had an early unplanned readmission died, compared to none in the “No early unplanned readmission” group (p=0.008) and 6 developed SBP in the “Early unplanned readmission” group, compared to 1 in the other group (p=0.027).

In comparison with “No early unplanned readmission”, “Early unplanned readmission” patients had higher care needs, with a greater number of admissions per month (p=0.001), total length of hospital stay per month (p=0.004) and number of paracenteses per month (p<0.001) (Table 7.3).

Table 7.3. Use of hospital services for “No early unplanned readmission” and “Early unplanned readmission”.

Clinical parameter, median (IQR)	“No early unplanned readmission” (n=19)	“Early unplanned readmission” (n=14)	p-value
Length of index admission hospital stay (days)	6.0 (3.0-11.0)	3.0 (1.0-15.3)	0.44
Total length of hospital stay during the study (days)	10.0 (6.0-26.0)	42.5 (15.8-77.8)	0.011
Total length of hospital stay per month (days)	1.5 (0.8-3.9)	10.7 (2.7-19.3)	0.004
Number of admissions per month	0.2 (0.1-0.5)	1.0 (0.4-2.0)	0.001
Number of paracenteses during the study period	2.0 (1.0-6.0)	7.0 (3.0-17.0)	0.001
Number of paracenteses per month	0.4 (0.1-0.7)	2.2 (0.7-3.6)	<0.001

7.4 Discussion

Although chronic liver disease is not currently a National Health Priority Area, there is increasing concern about the growing impact of liver disease on the health of Australians and the health care system. This study was undertaken to examine the use of hospital services by patients with cirrhosis and ascites, and to identify factors that may help detect those at risk of early readmission and with higher care needs. The present study indicated that decompensated chronic liver disease is associated with very high use of hospital services, with a number of admissions unplanned.

Chronic liver disease has a substantial latency period, during which affected individuals remain relatively asymptomatic despite progressive hepatic fibrosis and development of cirrhosis. Ascites is usually the first sign that cirrhosis has progressed to a decompensated phase.[278,402] The median survival time of patients with decompensated cirrhosis is around 2 years and, not unexpectedly, around one-quarter of our cohort died during the study. Over a 12 month period, patients in this study had frequent and prolonged hospital admissions, illustrating the high morbidity, mortality and resource utilisation of this patient population.

These findings are consistent with previous reports that medical care for decompensated cirrhosis and ascites is complex and patients with CLD often have comorbidities that increase the burden of illness and use of health care resources.[509,510] As evidenced by this study, patients are often prescribed multiple medications, many of which require dosage adjustments or titration based on clinical response, side-effects or laboratory follow-up. A recent study demonstrated that the number of medications on discharge was a risk factor for hospital readmission among patients with decompensated cirrhosis.[387] Although the precise reason for this was not established, it is likely contributed to by frequent dosage adjustments and potential compliance issues related to factors such as depression[511] and hepatic encephalopathy, which were common in this patient cohort. Our data suggests that comorbidities such as prior heavy or current alcohol consumption and diabetes may also contribute to early readmission. Diabetes and alcohol-related liver disease have previously

been identified as risk factors for frequent readmissions in patients with cirrhosis.[388] It has been suggested that diabetes is a risk factor for hepatic encephalopathy, [512,513] a factor more prevalent in our patients with early readmission. The role of alcohol use in early re-hospitalization emphasises the importance of assessing alcohol histories[514] and prompt referral to alcohol and drug treatment services.

This study demonstrated that patients with early unplanned readmissions experienced more hospital admissions, with longer hospital stays. Deterioration in liver or renal function or development of SBP may identify a subgroup of patients requiring more intensive follow-up and implementation of prophylactic interventions. MELD, a validated score that predicts survival in CLD patients, has been reported to predict early readmission following hospital discharge.[387,515] MELD is based on 3 objective, quantitative variables: serum bilirubin, international normalised ratio of the prothrombin time and serum creatinine.[383] In the current study patients with early unplanned readmission had higher MELD scores compared to subjects with no early readmission. There was also a significant difference for both haematocrit and haemoglobin levels between the two groups, which may be due to hemodynamic abnormalities in advanced cirrhosis or result from other complications of decompensated cirrhosis.

The majority of hospital readmissions during the study period were for management of ascites. Determining the specific factors contributing to readmission for ascites (e.g. poor efficacy or noncompliance with medical therapy, progression of liver disease or medication side-effects) was beyond the scope of the current study and should be a focus of future research. It has been speculated that one-quarter of hospital readmissions may be prevented by better patient understanding of their medication regime or more intensive outpatient monitoring.[387] Unfortunately a recent Australian pilot study involving intensive CLD patient monitoring after discharge did not show a reduction in occupied bed days or other secondary end-points of hospital use.[275] The findings did however suggest an improved *approach* to hospital use, with an increase in planned admissions and increased attendance rate at outpatient

care. Prospectively this may lead to better management and compliance with medications, and reduce use of emergency and radiological services. Improved coordination of patient care and specialist involvement[516] should provide a more efficient, less resource intensive approach to management of ascites, resulting in better patient outcomes[517,518] and cost savings.

This study likely reflects the patients and practices in other Australian tertiary care hepatology centres, but may not represent centres with less specialised services. The study was limited to one calendar year with 41 patients recruited during this period, thus only simple univariate statistical analyses were possible. In addition, statistical significance may have not been achieved for some clinical parameters due to the cohort size and because 6 of the patients received a liver transplant during the study period, all of whom had improved outcomes.

In conclusion, patients with cirrhosis and ascites comprise a relatively small population who account for a substantial use of hospital services, due to frequent admissions that are often unplanned. Management of these patients is complex and markers of disease severity may help identify patients who are at increased risk of poorer outcomes. The present study supports the need to consider alternative or adjunct models of care for this patient cohort to determine whether more intensive patient monitoring and coordination of patient care will reduce unplanned hospital admissions and result in reduced health care costs and pressure on emergency services.

CHAPTER 8

ASCITES MICROBIAL BURDEN AND IMMUNE CELL PROFILE ARE ASSOCIATED WITH POOR CLINICAL OUTCOMES IN THE ABSENCE OF OVERT INFECTION

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Contributor	**Statement of contribution**
Kevin Fagan *DM candidate*	Conceived and designed study (30%) Collected data and samples (100%) Laboratory work (45%) Data analysis (30%) Wrote the paper (45%) Edited the paper (13%)
Geraint Rogers *Microbiologist*	Data analysis (20%) Wrote the paper (5%) Edited the paper (13%)
Michelle Melino *Scientist*	Laboratory work (15%)
Dionne Arthur *Scientist*	Laboratory work (10%)
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Mark Morrison *Scientist*	Advice regarding next generation sequencing Edited the paper (2%)
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Abstract

Bacterial infections, most commonly spontaneous bacterial peritonitis in patients with ascites, occur in one third of admitted patients with cirrhosis, and account for a 4-fold increase in mortality. Bacteria are isolated from less than 40% of ascites infections by culture, necessitating empirical antibiotic treatment, but culture-independent studies suggest bacteria are commonly present, even in the absence of overt infection. Widespread detection of low levels of bacteria in ascites, in the absence of peritonitis, suggests immune impairment may contribute to higher susceptibility to infection in cirrhotic patients. However, little is known about the role of ascites leukocyte composition and function in this context. We determined ascites bacterial composition by quantitative PCR and 16S rRNA gene sequencing in 25 patients with culture-negative, non-neutrocytic ascites, and compared microbiological data with ascites and peripheral blood leukocyte composition and phenotype. Bacterial DNA was detected in ascitic fluid from 23 of 25 patients, with significant positive correlations between bacterial DNA levels and poor 6-month clinical outcomes (death, readmission). Ascites leukocyte composition was variable, but dominated by macrophages or T lymphocytes, with lower numbers of B lymphocytes and natural killer cells. Consistent with the hypothesis that impaired innate immunity contributes to susceptibility to infection, high bacterial DNA burden was associated with reduced major histocompatibility complex class II expression on ascites (but not peripheral blood) monocytes /macrophages. These data indicate an association between the presence of ascites bacterial DNA and early death and readmission in patients with decompensated cirrhosis. They further suggest that impairment of innate immunity contributes to increased bacterial translocation, risk of peritonitis, or both.

8.1 Introduction

Infections are responsible for much of the morbidity, mortality and resource utilization in patients with decompensated cirrhosis[519,520]. Bacterial infections, most commonly spontaneous bacterial peritonitis (SBP) in patients with ascites, occur in one-third of admitted patients with cirrhosis, and account for a 4-fold increase in mortality[521], but absence of clinical signs of infection is frequent and may delay diagnosis and treatment. Less than 40% of ascites infections are culturable, requiring initiation of empirical antibiotic treatment. The mortality rate after infection in cirrhosis remains high (28.6% at 1 month, 63% at 1 year) and has not changed substantially over recent decades[521]. However, culture-independent studies suggest bacteria are commonly present, even in the absence of overt infection. A combination of 16S rRNA gene sequencing and quantitative PCR was recently used to show that ascitic fluid from cirrhotic patients comprises a continuum from low-level bacterial colonization in the absence of a neutrophil response, through to clinically significant and severe SBP[522]. Although substantial variation in the bacterial species detected was observed between patients, microbiota community membership and structure correlated with differences in ascitic fluid neutrophil count and patient Child-Turcotte-Pugh (CTP) class[522]. The widespread detection of low levels of bacteria in ascites in the absence of peritonitis suggests first, that bacterial translocation to the peritoneal cavity is a common process, and second, that the entry of bacteria into this site may not be sufficient to give rise to SBP. Here, host immune impairment may also contribute to the risk of SBP in some cirrhotic patients. However, little is currently known about the role of ascites leukocyte composition and function in this context.

Innate immune cells, especially monocytes/macrophages, represent the first line of defence against microbes. Various defects in peripheral monocytes have been described in chronic liver disease (CLD)[520], including in anti-bacterial effector functions, similar to the “immune paralysis” observed in sepsis. Monocyte deactivation in patients with decompensated cirrhosis directly influences outcomes, and is a tractable therapeutic target[520,523]. However, monocyte deactivation is likely to change over time, and differ between anatomical sites. Ascitic fluid provides a unique portal through which immune

function can be assessed at the site of infection, but ascites leukocytes have been surprisingly little studied. Moreover, ascitic fluid has been reported to contain (unidentified) immune inhibitory factors[524]. The extent of immunoparalysis in ascites, and the relative contribution of cell intrinsic and cell extrinsic factors, is not known.

The first aim of this study was to quantify bacterial DNA in ascitic fluid, in order to determine whether bacterial burden is associated with clinical outcomes, including infection, survival or incidence of decompensation events (upper gastrointestinal bleeding, hepatic encephalopathy, hepatocellular cancer). The second aim was to characterise ascitic fluid and peripheral blood leukocytes, to determine the extent to which immune phenotype is site-specific, and its relationship to microbial burden, clinical parameters and outcomes.

8.2 Experimental Procedures

8.2.1 Patients and clinical data

Ascitic fluid and matched peripheral blood samples were obtained from 25 patients with decompensated cirrhosis undergoing paracentesis. Informed written consent was obtained from each patient and the protocol was approved by the Metro South Health and The University of Queensland Human Research Ethics Committees. Standard biochemical and serological assays, liver imaging and histological assessment of a liver biopsy (if performed) were used to confirm diagnosis of liver disease and cirrhosis. Liver disease severity was evaluated using the CTP classification[507], and Model for End-Stage Liver Disease (MELD)[508]. Patient medical records were reviewed to obtain demographic details, previously diagnosed liver disease and other medical conditions, medications and history of tobacco and alcohol use for each patient. Current alcohol use was defined as ‘significant’ if the patient consumed greater than the threshold of alcohol likely to cause liver injury, based on epidemiological data (140g/week for women, 210g/week for men)[9]. Heavy alcohol use was defined as ≥350g/week for women and ≥420g/week for men, for >6 months. Blood was drawn at the time of paracentesis for routine laboratory tests/blood cultures, or processed and stored at -20ºC for analysis of

C-reactive protein (Beckman DXC800), and procalcitonin (Mini Vidas). The serum-ascites albumin gradient (SAAG) was calculated by subtracting the albumin concentration of the ascitic fluid from the albumin concentration of a serum specimen obtained on the same day. SBP was defined as an ascitic fluid polymorphonuclear leukocyte count ≥250/mm^3. Clinical outcome data for the 6 months after the paracentesis (survival, liver transplantation, readmission, incidence of SBP or decompensation events) were obtained from medical records.

8.2.2 Cell isolation and DNA purification from ascites samples

14ml Ascites fluid was centrifuged at 5000x*g* for 10 minutes in sterile 15ml conical tubes. DNA was extracted from cell pellets using the QIAamp DNA Mini kit in accordance with the manufacturer's instructions (Qiagen, Venlo, NL), resuspended in 100µl sterile water, and quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA).

8.2.3 Assessment of bacterial density

A real-time PCR assay that amplifies a 286 base pair region of the V4 region of the 16S rRNA gene was used to quantify bacterial DNA. PCR primers used were 517F (5′-GCCAGCAGCCGCGGTAA-3′) and 803R (5′-CTACCRGGGTATCTAATCC-3′)[525]. The total volume for each reaction was 20µl, containing 1x SYBR Select Master Mix (Applied Biosystems, Mulgrave, Australia), 1µl of 0.5µM forward and reverse primers and 50ng of template DNA. PCR was performed using an Mx3000P thermal cycler (Agilent Technologies, Santa Clara, USA) with the following cycling conditions: 50ºC for 2 min, 95ºC for 2 min, and 40 thermal cycles of 95ºC for 15 sec; 57ºC for 30 sec; 60ºC for 30 sec. A final step of 95ºC for 1 min and a dissociation curve protocol from 55º to 95º were performed. Phosphate buffered saline (PBS) that had been through the DNA extraction process was used as a negative control. PCR threshold cycles were used to calculate the amount of bacterial DNA in ascitic fluid (ng/µl) using a standard curve generated from purified *Escherichia coli* DNA and converted to an estimate of colony forming units (CFU)/ml.

8.2.4 16S rRNA amplicon sequencing

Sequencing of the V4 region of the 16S rRNA gene amplicon was carried out by MrDNA.com (Texas, USA) using primers 515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACVSGGGTATCTAAT-3′ primers[525]. In brief, a single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was performed under the following conditions: 94°C for 5 minutes, followed by 28 cycles of: 94°C for 30 sec, 53°C for 40 sec, and 72°C for 1 min. Amplification was followed by a final elongation step at 72°C for 5 minutes. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Sequencing was performed using the Roche 454 FLX titanium instruments and reagents according to manufacturer's instructions[526]. Sequencing analysis was performed as previously described[527,528].

8.2.5 Cell isolation and flow cytometric analysis

Cells were pelleted from ascites fluid at 500x*g* for 5 minutes, washed once with low glucose Dulbecco's modified eagle medium (Invitrogen), and resuspended in freezing medium (10% DMSO (Sigma-Aldrich, Castle Hill, Australia), 50% fetal bovine serum (FBS, Invitrogen)), and stored in liquid nitrogen. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll density centrifugation, resuspended in freezing medium, and stored in liquid nitrogen. For flow cytometric analysis, 0.5-1x10^6 PBMC or ascites cells were stained in 50µl PBS/2%FBS with a panel of antibodies comprising HLA-DR-FitC, CD56-PE, CD66B-PerCp.Cy5.5, CD3-AF700, CD19-APC, CD16-APC.H7, CD14-BV421 (leukocyte panel) or HLA-DR-FitC, CD11C-PE, CD163-PerCp.Cy5.5, CX3CR1-PE.Cy7, CCR2-APC, CD16-APC.H7, CD14-BV421 (monocyte panel) or single antibodies for compensation (Biolegend, San Diego, CA, USA or Becton Dickinson, Franklin Lakes, New Jersey, USA). Flow cytometry analysis was performed on a Gallios flow cytometer and data analysed using Kalluza (Beckman Coulter, Brea, CA, USA). Cell populations were gated based on unstained controls and single positive controls. Additionally, fluorescence-minus-one controls were used to verify background fluorescence levels during protocol development. Negative myeloid and lymphoid populations were gated

separately as myeloid (SSC^{Hi}) cells exhibited increased autofluorescence compared to lymphocytes. $CD14^{Hi}$ cells formed an obvious, distinct population in all donors, facilitating their gating. A second, distinct SSC^{Hi} population was observed in the majority of donors, which apparently comprised $CD14^{Low}$ and $CD14^{Negative}$ cells, based on negative controls, but could not be clearly distinguished on the basis of CD14 expression.

8.2.6 Statistical analysis

Statistical analysis of clinical and flow cytometry data was performed in Prism 6.04 (GraphPad, La Jolla, California, USA). Spearman's correlation was used to compare continuous variables, and Mann Whitney U tests were used for group comparisons. A significance threshold of $p<0.05$ was used. Several statistical tools were used to assess whether relationships existed between bacterial community composition and clinical factors. Measures of bacterial community diversity (genus richness, Simpson index, and Shannon index) were assessed using PAlaeontological STatistics, version 3.01 (PAST), available from the University of Oslo (http://folk.uio.no/ohammer/ past). Non-metric Multidimensional scaling (NMS) based on Bray-Curtis (BC) similarity measures, one-way ANOSIM tests, were used to assess whether bacterial community composition differed significantly between groups according to categorical variables (etiology, previous SBP, prophylactic antibiotic use, antibiotic treatment within preceding 14 days, and 6 month mortality) using PAST.

8.3 Results

8.3.1 Patient characteristics at paracentesis

Twenty five patients undergoing paracentesis for ascites secondary to chronic liver disease of various etiologies, principally alcohol and hepatitis C virus (HCV) infection, were recruited (Table 8.1). The median age of the cohort was 55.4 years, 76% were male and 96% Caucasian. Eighteen patients admitted previous heavy alcohol consumption, although only 7 reported significant alcohol consumption during the prior year, 3 within the 2 weeks before the current paracentesis. The median number of prior paracenteses was 4. Over

the 6 month follow-up period 2 patients developed their first episode of SBP, 9 patients died (1 during admission for SBP, 1 from pneumonia) and 3 received a liver transplant. Seven patients died with decompensated cirrhosis, however the contribution of bacterial infections to their decompensation and death could not be accurately determined. There were 64 hospital admissions over the 6 month follow up period, in addition to 44 presentations to the day procedure unit for paracentesis.

Table 8.1. Patient demographic data and comorbidities at the time of ascitic fluid collection and outcomes during the 6 months follow up.

	Patient Cohort n=25
At Paracentesis	
Age (years) median (IQR)	55.4 (50.0-64.1)
Gender (n, % male)	19 (76)
Ethnicity (n, % Caucasian)	24 (96)
Etiology of cirrhosis (n, %)	
Alcohol	11 (44)
Hepatitis C virus	9 (36)
Other[a]	5 (20)
Previous SBP (n, %)	8 (32)
Previous evidence of gastroesophageal varices (n, %)	12 (48)
Previous evidence of hepatic encephalopathy (n, %)	7 (28)
Hepatocellular carcinoma (n, %)	3 (12)
Previous heavy[b] alcohol consumption (n, %)	18 (72)
Significant[c] alcohol consumption in last year (n, %)	7 (28)
Days since previous paracentesis, median (IQR)	147 (4-856)
Number of previous paracenteses, median (IQR)	4.0 (0-29)
Outcomes (6 months)	
Death (n, %)	9 (36)
Developed SBP (n, %)	2 (8)
Liver transplant (n, %)	3 (12)

Table 8.1 notes. [a]Other: Non-alcoholic fatty liver disease (n=1), cryptogenic cirrhosis (n=1), primary biliary cirrhosis (n=1), primary sclerosing cholangitis (n=1), autoimmune hepatitis (n=1); [b]Previous heavy alcohol use was defined as ≥350g/week for women and ≥420g/week for men, for >6 months; [c]Significant alcohol was defined as >140g/week for women and >women and >210g/week for men.

8.3.2 Bacterial DNA burden in ascitic fluid in the absence of overt infection is associated with poor clinical outcomes

Bacterial 16S rRNA DNA (hereafter '16S') was detectable in 23 of 25 patients, at levels equivalent to 0.09-1.9 ng/µl *E.coli* DNA, which would equate to approximately 10^3-10^5 *E.coli* CFU/ml (CFU equivalents/ml, hereafter 'CFU/ml'). Clinical histories of this cross-sectional cohort were diverse; many patients had prior events expected to influence gut and/or ascites microbiota, including previous paracenteses, SBP, and antibiotic treatment (summarised in Figure 8.1, relative to ascites bacterial DNA levels). Two patients were diagnosed with SBP (ascites neutrophil count ≥250/mm^3) 2 days prior to paracentesis, but none of the patients had an ascites neutrophil count ≥250/mm^3, positive bacterial culture, or evidence of extra-abdominal infection at the time of ascitic fluid collection for this study. Prior to fluid collection 8 patients had previously had SBP, including the 2 diagnosed in the 2 days before, but only 2 had organisms cultured using conventional microbiological culture techniques (*E.coli* and *Streptococcus mitis*). The median length of time between patients' most recent episode of SBP and the paracentesis for this study was 86 days (IQR 22.3-174.3). Sixteen patients (64%) had received antibiotics within 2 weeks of the paracentesis: SBP prophylaxis (n=7); intravenous broad spectrum antibiotics <48 hours prior to sample collection (n=7) or ≤7 days prior to sample collection (n=2); other oral antibiotic courses (n=3, 1 for *Helicobacter pylori* eradication (7 days), 1 for cellulitis prophylaxis (120 days) and 1 was patient directed, taking antibiotics 1 day before paracentesis). There was no significant difference in 16S levels between patients who had antibiotics during the previous 2 weeks and those who had not (p=0.28, Figure 8.2A).

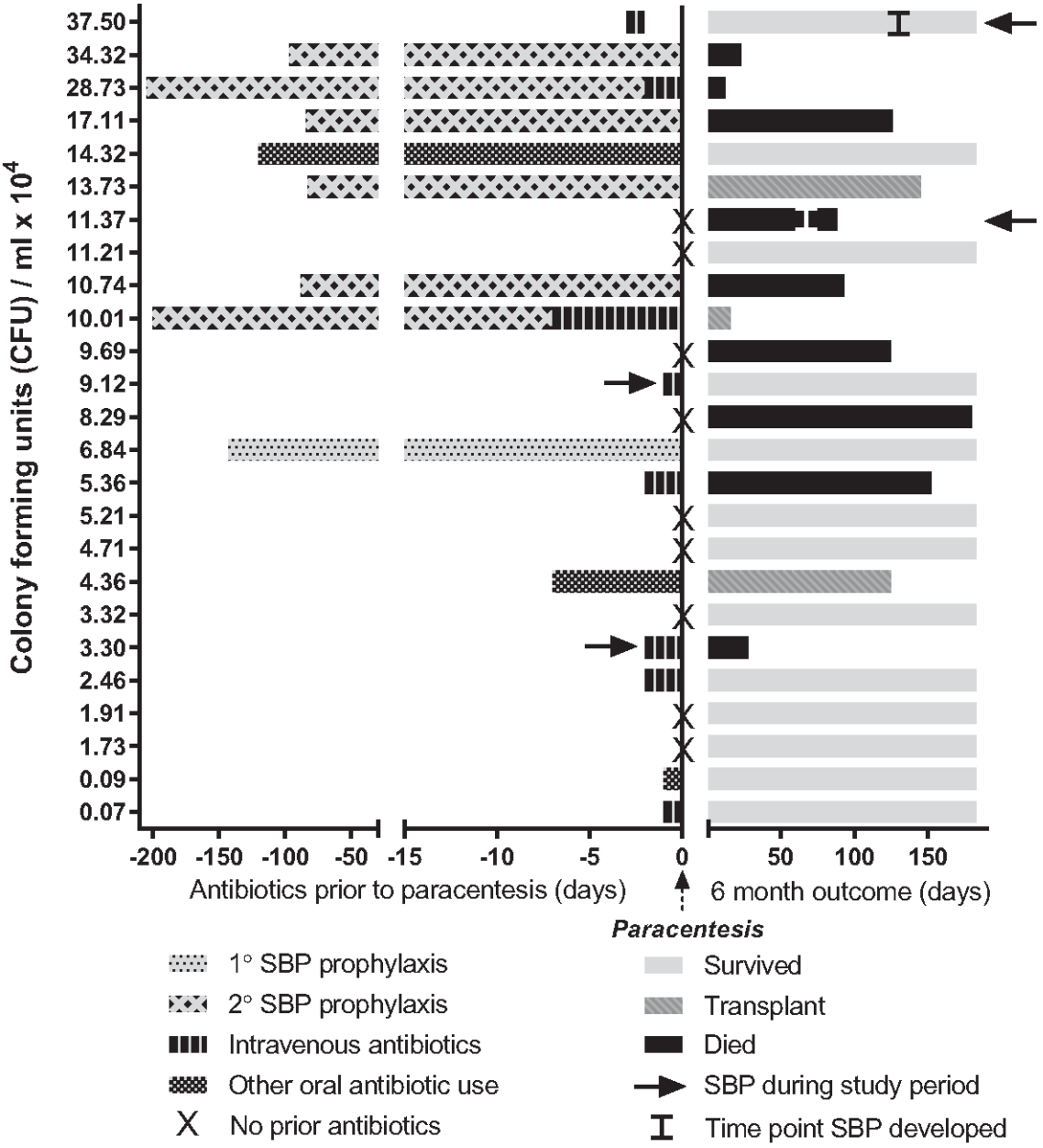

Figure 8.1. Ascites cohort antibiotic history and 6-month outcomes. Antibiotic treatment history prior to the study paracentesis and 6 month outcomes are depicted for each patient, in relation to their ascitic bacterial DNA burden (CFU/ml).

Bacterial burden was weakly correlated with the number of neutrophils in the ascitic fluid (r_s=0.5, p=0.012) (Figure 8.2B), but did not correlate with serum markers of infection/inflammation (C reactive protein (p=0.44) or procalcitonin (p=0.52)). Patients with a prior diagnosis of SBP had significantly higher CFU/ml (p=0.027, Figure 8.2C), particularly those taking oral prophylaxis. Bacterial burden was negatively correlated with ascites total protein level (r_s=-0.42, p=0.045, Figure 8.2D), but not with serum markers of renal impairment (urea, creatinine, sodium) or liver failure (bilirubin or CTP score).

As highlighted in Table 8.1 and Figure 8.1, clinical outcomes for patients with ascites were poor. Bacterial DNA burden was associated with a shorter time to readmission (r_s=-0.50, p=0.024, Figure 8.2E), and was significantly higher in patients who died or developed SBP within 6 months (p=0.006, Figure 8.2F). Although 2 patients had a diagnosis of SBP 2 days prior to inclusion into the study, they were culture negative and non-neutrocytic at the time of sampling (19 and 33 PMN/ml) and there was nothing in their clinical features or history to distinguish them (6 other patients had previous SBP, 7 were treated with IV antibiotics around the time of sampling). Analysing the data after excluding these patients produced the same results reported here.

The most significant clinical parameters associated with death or subsequent development of SBP were mean arterial blood pressure (MAP, p=0.007) and low levels of ascites total protein (p=0.021), which was also associated with high bacterial burden (Figure 8.2D). There was a trend towards significantly worse outcomes for patients who had a previous episode of SBP (p=0.056), but there were no differences for markers of renal or liver failure mentioned above. Prescription of propranolol, lactulose, proton pump inhibitors (PPI) or diuretics did not differ between the 2 cohorts (all p>0.3).

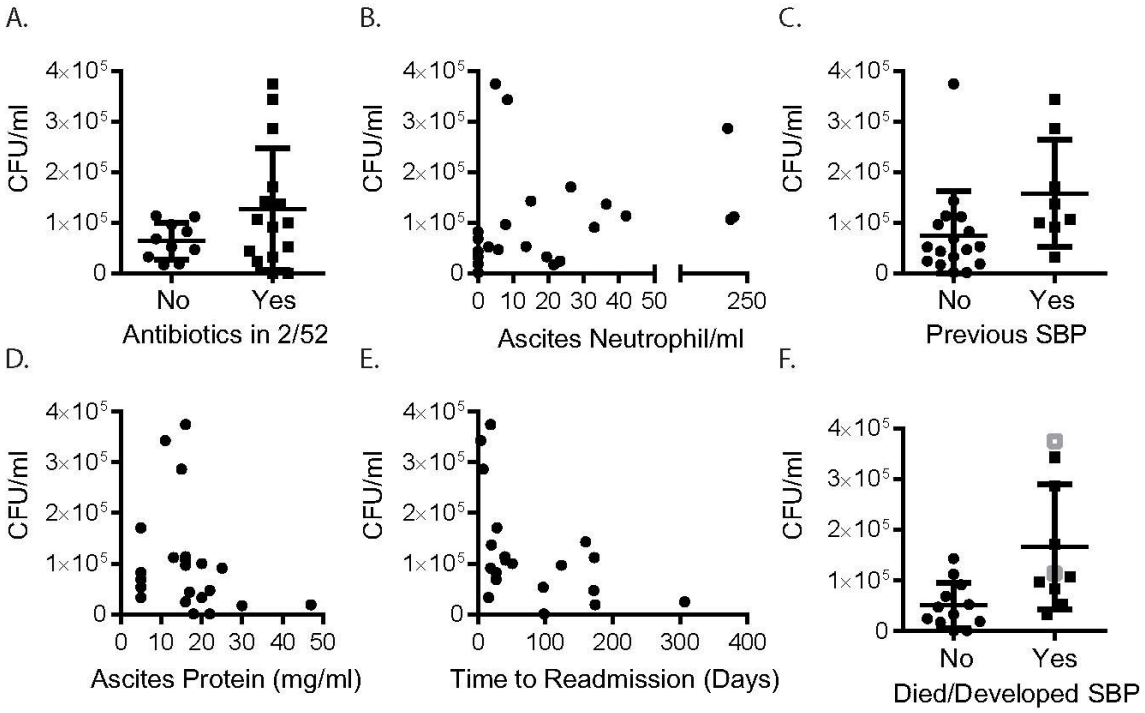

Figure 8.2. Bacterial DNA burden in ascitic fluid is associated with poor clinical outcomes. **(A)** Ascitic bacterial DNA burden in patients who had received antibiotics within the previous 2 weeks (2/52) (p=0.28). **(B)** Correlation between bacterial DNA burden and the number of neutrophils/ml ascitic fluid (r_s=0.5, p=0.012). **(C)** Bacterial DNA burden in patients with a previous history of SBP (p=0.027). Correlation between bacterial DNA burden and **(D)** ascites total protein content (r_s=-0.42, p=0.045) and **(E)** time to hospital readmission (r_s=-0.50, p=0.024). **(F)** Bacterial DNA burden in patients who survived and those who died (black squares) or developed SBP (grey squares, # developed SBP and died, p=0.006).

8.3.3 Ascites bacterial community composition

16S rRNA gene amplicon sequences were used to assess bacterial community composition. Bacterial phyla comprised both Gram positive and Gram negative taxa. In keeping with previous studies, the most commonly detected phylum was Proteobacteria (median relative abundance 28.6%), followed by Actinobacteria (14.3%), Firmicutes (7.7%), and Bacteroidetes (3.4%), with no other single phylum representing more than a median value of 0.39% of total

abundance (Figure 8.3). When assessed at the genus level, samples had a median taxon richness of 23 (range: 9-96, IQR15-37.5). The most commonly detected genera included *Streptococcus* (15/25 patients), *Porphyromonas* (12/25 patients), and *Enterobacter* (12/25 patients). A significant correlation between relative genus abundance and frequency of detection (the number of patients a genus was detected in) was observed (Spearman's r=0.59, p<0.0001). Bacterial community composition differed substantially between patients, with a genus level mean BC score of 0.06 ± 0.06 (where a score of 1 indicates identical communities, and 0 indicates no similarity). Further, no significant clustering was identified by NMS, suggesting a stochastic community assembly. Neither were statistically significant correlations identified between bacterial community composition, or community diversity measures and categorical or continuous clinical variables.

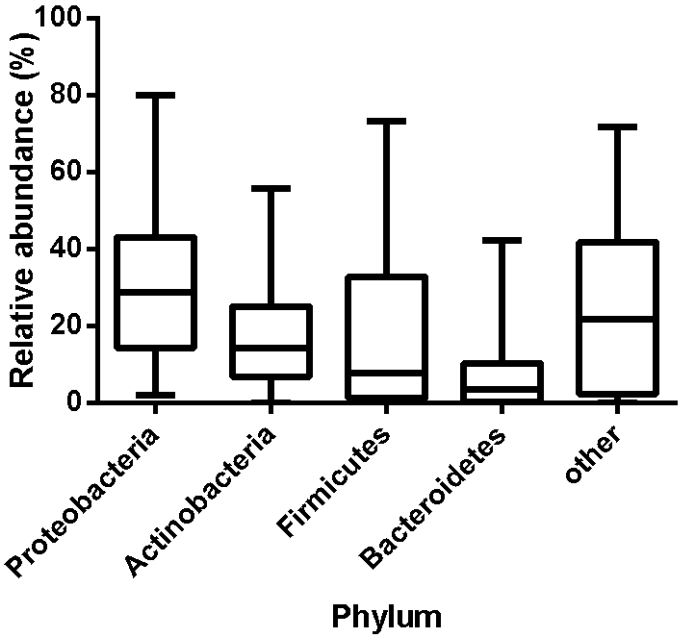

Figure 8.3. Distribution of bacterial phyla identified by 16S sequence analysis of ascites bacterial DNA. Boxes represent median values and error bars show interquartile range.

8.3.4 Ascites fluid leukocyte composition and phenotype

Ascites immune cells from 18 patients were profiled using a panel of markers to enumerate T cells (CD3+), B cells (CD19+), monocytes/macrophages (CD14+), and natural killer (NK) cells (CD56+), as well as staining for CD16, HLA-DR and the granulocyte activation marker CD66B to further phenotype cells of interest (Figure 8.4). These lineage markers typically accounted for >95% of ascites cells. The most abundant leukocytes in ascites fluid were generally $CD14^{High}$ macrophages (median 38.6%), with lower numbers of $SSC^{High}/CD14^{Low/-ve}$ myeloid cells (median 11.2%), T cells (median 19.9%), NK cells (median 6.1%), and B cells (median 1.1%) (Figure 8.5A). Unlike peripheral blood monocytes, a minority of which express CD16 (approximately 10%), the majority of ascites $CD14^{High}$ cells co-expressed CD16; and essentially all expressed HLA-DR. $CD16^{-}$ and $CD16^{+}$ macrophage populations were not clearly discernible (Figure 8.4C), however there was a broad spectrum of CD16 expression. $SSC^{High}/CD14^{Low/neg.}$ cells were subdivided on the basis of HLA-DR expression. $CD14^{Low/neg.}/HLADR^{neg.}$ cells were typically $CD16^{+}$, with a variable proportion of $CD66B^{+}$ cells (activated granulocytes). The $CD14^{Low/neg.}/HLA\text{-}DR^{+}$ population may contain monocytes/macrophages with low HLA-DR expression; CD16 expression in the population varied, but all were $CD66B^{Neg.}$ (Figure 8.4D). We further phenotyped ascites myeloid cells from 7 patients, using a panel of markers that distinguish blood monocyte subsets. Greater than 90% of ascites $CD14^{High}$ macrophages co-expressed CCR2, CX3CR1, CD163 and CD11c. $CD14^{Low/-neg.}$ cells were CX3CR1+, and a proportion expressed CCR2, and CD163 and CD11c at low levels, consistent with the presence of a $CD14^{Low/neg.}$ monocyte/macrophage population (Figure 8.4E).

The ascites lymphocyte compartment was not characterised in detail in this study, however we did observe a high proportion of T cells expressing the NK cell-associated receptor CD56+ in a subset of patients (Median 20%, (range 1-50%), which can be induced by T cell receptor stimulation, and has been shown to mark a subset of T cells with major histocompatibility complex-unrestricted cytotoxicity[529] (Figure 8.4B). Ascites NK cells could be subdivided into $CD56^{High}/CD16^{-}$, immunoregulatory cells, and $CD56^{Low}/CD16^{+}$, cytotoxic, cells,

however their relative proportions were extremely variable ($CD56^{High}/CD16^{-}$ Median 8% (range 0-47%)) (Figure 8.4B). $CD56^{Low}/CD16^{+}$ cells comprise the majority of peripheral blood NK cells (90%), whereas $CD56^{High}/CD16^{-}$ NK cells, thought to be the direct precursors of $CD56^{Low}/CD16^{+}$ cells, dominate secondary lymphoid organs and tissues[530]. With the exception of NK cells, leukocyte frequency in ascites fluid was not related to their proportions in peripheral blood collected at the time of paracentesis (data not shown).

8.3.5 High ascites bacterial burden is associated with reduced macrophage HLA-DR expression

The presence of monocytes/macrophages ($CD14^{+}$/HLA-DR^{+}) in ascites fluid was associated with lower ascites neutrophil numbers and a trend towards lower bacterial burden (r_s=-0.59, p=0.011 and r_s=-0.41, p=0.081 Figure 8.5B and data not shown). Consistent with the hypothesis that innate immune function is impaired in patients with cirrhosis, HLA-DR expression on ascites $CD14^{Hi}$/HLA-DR^{+} monocytes/macrophages inversely correlated with bacterial DNA levels (r_s=-0.48, p=0.04, Figure 8.5C). Similar to the association between high bacterial DNA levels and low ascites total protein content, macrophage HLA-DR expression inversely correlated with the serum ascites albumin gradient (SAAG, rs=-0.59, p=0.01 Figure 8.5D), indicative of low ascites total protein and portal hypertension. Like bacterial DNA burden, low macrophage HLA-DR expression was associated with shorter time to readmission (rs=0.546, p=0.036, Figure 8.5E). There was a non-significant trend towards lower macrophage HLA-DR expression in patients who had previous episodes of SBP, and in those who died during the 6 months follow-up. Interestingly, patients' ascitic macrophage HLA-DR expression was not related to HLA-DR expression on their peripheral blood monocytes. Although minor in proportion, B lymphocytes were the only cell type whose frequency was positively correlated with ascites bacterial burden (r_s=0.65, p=0.003), however B cell HLA-DR expression did not correlate with bacterial burden.

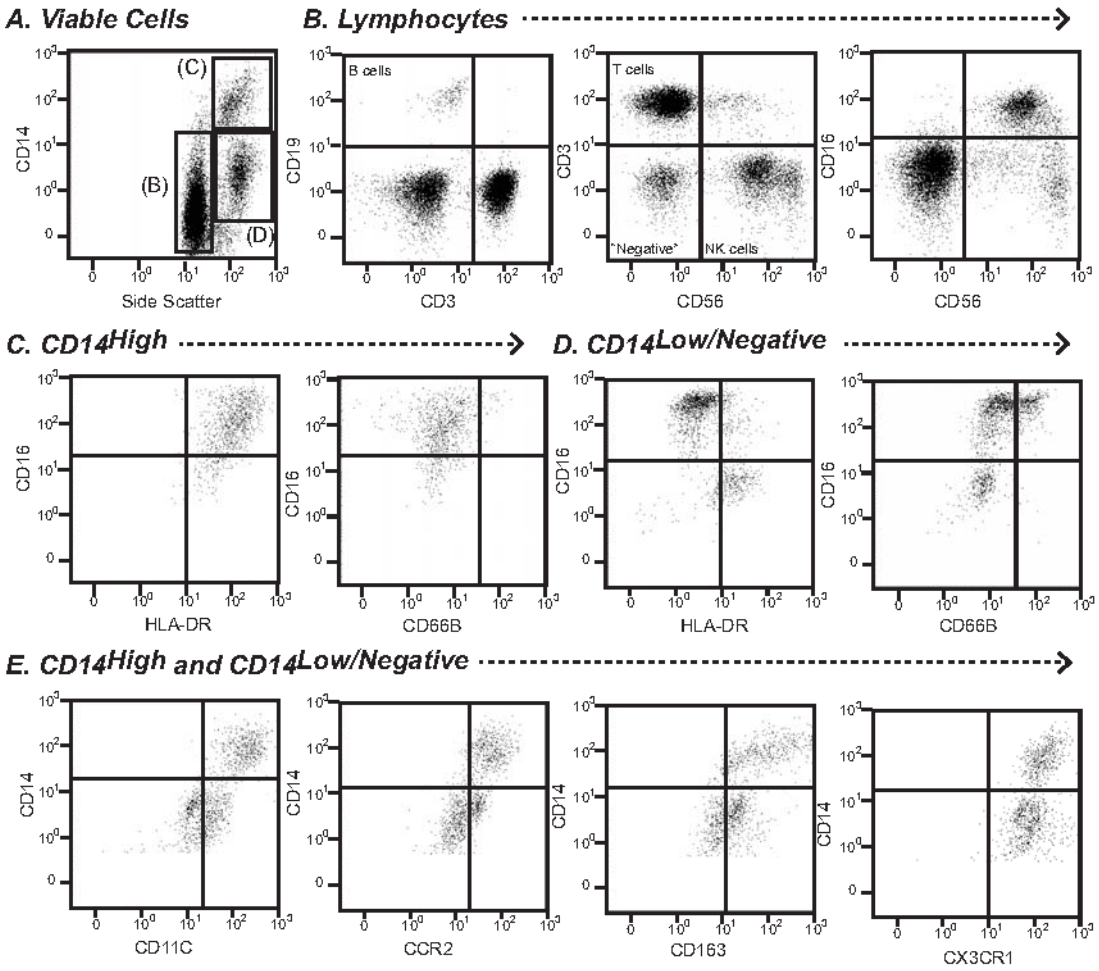

Figure 8.4. Flow cytometry gating strategy for ascites leukocyte characterisation. **(A)** Lymphocytes and myeloid cells were distinguished on the basis of side scatter properties and CD14 expression. CD14Hi and CD14 Low/^{negative} cells were further characterised for CD16, HLA-DR and CD66B expression (top right panels). Lymphoid cells were classified as B cells (CD19+), T cells (CD3+), and NK cells (CD56+/CD16+/-) (bottom left panels). **(B)** Myeloid populations were further investigated for surface CD11C, CCR2, CD163 and CX3CR1 expression.

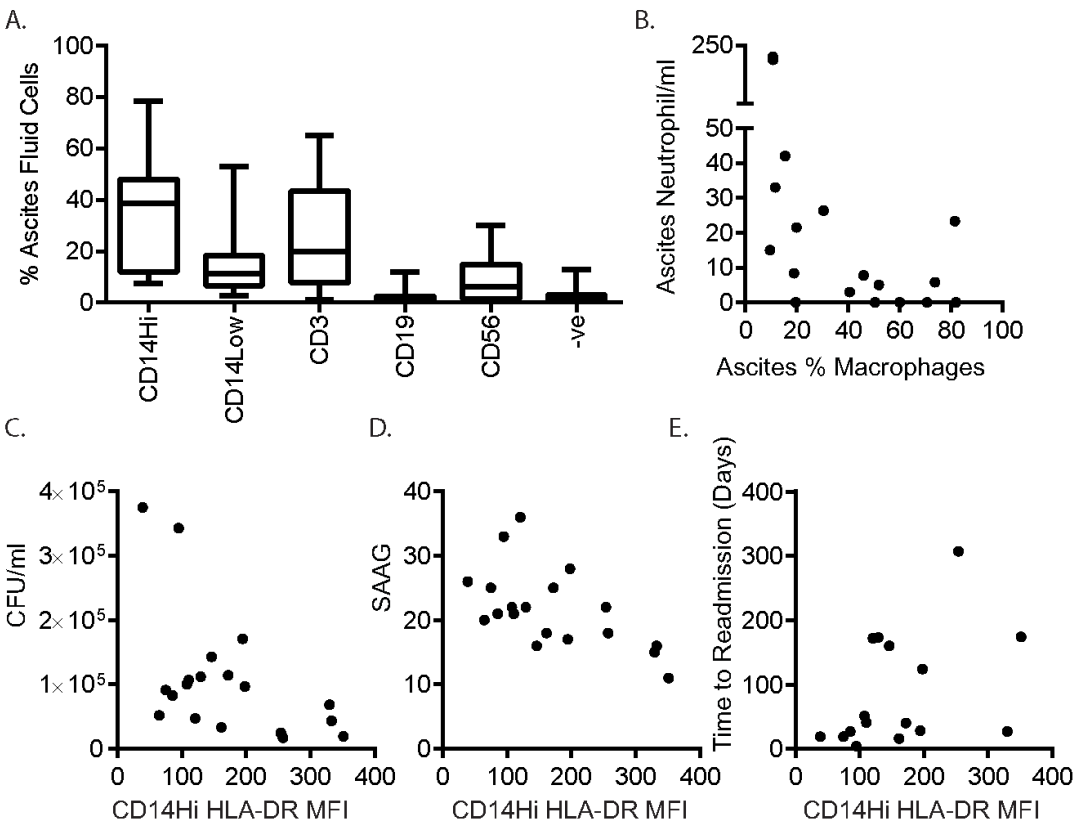

Figure 8.5. Ascites bacterial DNA burden associated with reduced HLA-DR expression on ascitic fluid macrophages **(A)** distribution of leukocyte lineages in ascites fluid (n= 18, midline represents median, box represents 25th-75th percentile, whiskers indicate minimum and maximum values, -ve indicates lack of staining for markers employed in this study) **(B)** Correlation between %CD14+ macrophages and neutrophil count in ascitic fluid. Correlation between surface HLA-DR expression on CD14+ ascites cells (MFI) and **(C)** ascites bacterial burden (CFU/ml), **(D)** serum ascites albumin gradient (SAAG) and **(E)** time to next hospital admission.

8.4 Discussion

Bacterial infection is a major cause of early death in patients with cirrhosis. Previous studies have reported the presence of bacterial DNA in ascites fluid, even in culture-negative and non-neutrocytic ascites[430,522], and confirmed at least some of this DNA is associated with viable bacteria[431]. However, the clinical significance of ascites bacterial DNA has not been widely studied. We report both the detection of bacterial DNA in a high proportion of culture negative, non-neutrocytic ascites fluid (23/25 patients) and a positive association between levels of bacterial DNA and poor clinical outcomes, including readmission, and death. Whether the presence of bacterial DNA in ascites fluid represents a sub-clinical or pre-clinical infection, or simply reflects the severity of other features of liver disease, such as portal hypertension or intestinal imbalance, is not clear. A previous report that ascites bacterial DNA was associated with short term mortality, but not with infection, would support the latter interpretation[441]. Moreover, portal hypertension has been suggested as a cause of intestinal permeability and bacterial translocation, and plasma levels of bacterial DNA were associated with systemic circulatory abnormalities in cirrhotic patients with ascites[531].

In keeping with previous studies, the ascites bacterial composition reported here comprises a broad phylogenetic range, including Gram positive and Gram negative species, with a predominance of Proteobacteria and a high relative abundance of Actinobacteria, Firmicutes, and Bacteroidetes. These phyla represent the four most commonly associated with the human microbiota, and those which typically dominate commensal communities in the gut and elsewhere. The relative abundance of the phyla detected in ascitic fluid differs from that seen in the gut of healthy individuals, where Bacteroidetes and Firmicutes typically dominate[323]. This distribution suggests that translocation of bacteria to the peritoneal cavity is not limited to those present in the gut, or that the gut microbiota in this patient population differs from that in healthy individuals. There is evidence to support each of these models. Qin and co-workers recently described intestinal dysbiosis in cirrhotic patients, and reported that the majority of patient-enriched species were of buccal origin, suggesting

translocation from the mouth to the gut occurs in cirrhosis[338]. It has also been shown that in patients with cirrhosis, there is shift in the composition of the gut microbiota, with a decrease in the relative abundance of Bacteroidetes and an increase in the relative abundance of Proteobacteria and Fusobacteria[336]. Analysis of detected bacteria at the genus level revealed the genera *Streptococcus*, *Porphyromonas*, and *Enterobacter* were commonly present, in keeping with translocation from the oral cavity or intestine. The absence of significant correlations between the bacterial composition of the samples analysed and clinical markers of disease is notable. NMS analysis indicated that there was low similarity between the profiles generated from individual patients. The absence of a consensus microbiota composition is likely to reflect the heterogeneous patient population, the multifactorial nature of cirrhosis, and the stochastic nature of bacterial translocation from areas of highly complex bacterial microbiota. Given this high degree of inter-individual heterogeneity, longitudinal studies in larger cohorts, with parallel sequencing of intestinal microbiota, will be required to investigate the relationship between ascites bacterial composition, immunity and clinical outcomes, including infection.

The common detection of low level bacterial DNA in ascites, and the previous reports of the detection of viable bacterial cells using culture-independent methodologies in patients without peritonitis, suggests that translocation of bacteria into the peritoneal cavity is necessary, but not sufficient, to trigger SBP. Impaired innate immune function in cirrhotic patients is likely to contribute to susceptibility to infection. Previous studies have reported low levels of HLA-DR expression on peripheral blood monocytes in critically ill patients with cirrhosis[532,533] and acute liver failure[534]. We observed reduced HLA-DR expression on ascites (but not peripheral blood) macrophages/monocytes, which was associated with increased ascites bacterial DNA burden. The presence of macrophages per se, however, correlated with reduced ascites neutrophils and bacterial DNA, suggesting that restoring macrophage function (e.g. HLA-DR expression) may be a viable therapeutic strategy. Diminished monocyte HLA-DR expression, which compromises T cell activation[535], is a

well-established biomarker of a transient, compensatory anti-inflammatory response syndrome that occurs in response to infection or systemic inflammation[536]. This post-inflammatory immunodeficiency, in which innate immune cells become refractory to further stimulation, prevents inflammation-induced injury, but can also predispose patients to lethal infection. The phenomenon is also known as endotoxin tolerance, although it is not specific to endotoxin, as other exogenous and endogenous stimuli can induce tolerance. The mediators and mechanisms of endotoxin tolerance are not fully understood, and different mechanisms underlie different aspects of the phenotype[537,538]. IFNγ and GM-CSF[538-540] have been reported to restore HLA-DR expression and phagocytic function in vitro and in vivo, including in patients with cirrhosis[539], however host inactivation of the inflammatory stimulus, in particular endotoxin, can also be required for recovery from the tolerant state[541]. The mechanisms underlying reduced HLA-DR expression in ascites, the duration of the phenotype and the functional implications warrant further investigation.

Clinical evidence supports the use of antibiotics for SBP treatment and prophylaxis, but how antibiotics impact on the gut and ascites microbiota is yet to be defined. Changing bacterial and resistance patterns in patients with cirrhosis have been attributed to the increasing use of antibiotic prophylaxis and invasive procedures, highlighting the importance of ensuring appropriate antibiotic use[542]. Long term use of broad-spectrum antibiotics may lead to an increase in pathogenic bacteria in the gut and increased antibiotic resistance[436,542,543]. Whether adjunct approaches such as pro- or pre-biotic treatment to restore gut homeostasis, or immunomodulatory approaches to boost immunity, without exacerbating immunopathology, could be useful in this cohort remains to be evaluated. Approaches that reduce the burden of endotoxin (or other inflammatory stimuli), or restore HLA-DR expression and immune function (e.g. IFNγ, GM-CSFor albumin treatment [539,544,545]) may be viable therapeutic strategies. Immune monitoring protocols are also needed to identify patients who may benefit from such interventions.

In conclusion, we report that the presence of ascites bacterial DNA in patients with decompensated cirrhosis and ascites is associated with early death and readmission, and is an indicator of impaired immunity, which may contribute to susceptibility to infection in these patients. Whether the presence of DNA, or other bacterial products, is indicative of a sub-clinical infection, or simply a persistent source of inflammatory stimuli that exacerbates liver pathology, remains to be clarified. Characterisation of ascites bacteria, their source and role in infection and inflammation, as well as the contribution of host immune factors, is crucial to developing effective treatment regimens, and minimising antibiotic resistance.

CHAPTER 9

FINAL DISCUSSION AND FUTURE PERSPECTIVES

The burden of CLD on the health care system is rising due to the increasing number of patients with advanced fibrosis. The development and validation of biomarkers for liver disease progression and risk factors associated, is vital to improving detection and management of patients with CLD, to enable better coordination of care, improve health outcomes and reduce health care costs. The aim of this thesis was to establish the need for biomarkers in patients with CLD and assess known and novel biomarkers proposed for the assessment of alcohol consumption, liver fibrosis and management of patients with cirrhosis and ascites.

The detection of risky alcohol consumption is important as it is both a primary and comorbid cause of liver injury and can significantly impact the patient's current and future care. Although the efficacy of structured screening questionnaire methods has been demonstrated[33], there is little or no data regarding their use for patients being evaluated for liver disease, a cohort of patients where risky drinking behaviour is likely and where detection will have significant benefits. Furthermore, over recent years there has been a substantial drive to improve preventative care by the Royal Australian College of General Practitioners[546], including assessment and management of risky alcohol use, but there is no published data regarding the quality and accuracy of alcohol histories on referrals to the hepatology clinic.

Alcohol histories documented in referral letters and medical records for patients seen in the hepatology clinic were therefore assessed and the need for a biomarker in the hepatology clinic evaluated. In addition, concordance between alcohol histories recorded during a hepatology consultation and those obtained by patient interview, including validated alcohol questionnaires (AUDIT[28], bMAST[47]), was assessed (Chapter 3). The findings were consistent with previous studies[20,455], demonstrating poor documentation and quantitation of alcohol consumption in referrals from general practitioners (GPs) or other specialty units. This is important since GPs have a key role as advocates for health promotion, and because lack of detection may have hindered earlier intervention. Hazardous alcohol consumption was prevalent in the study cohort, but despite this, follow-up documentation in the hepatology clinic was poor. The validated screening tools performed well for identifying patients with recent at-

risk drinking. There was good concordance between alcohol histories documented in the clinic and those obtained at interview, but the validated screening tools identified that in approximately 10% of cases, the documented alcohol consumption underestimated actual intake, with resultant clinical implications.

The study did not address why medical practitioners referring patients or reviewing them in the hepatology clinic did not use validated alcohol screening tools in their assessment. Studies have previously reported poor uptake of alcohol questionnaires[48,49] and the likely reasons for this[50,51,53,54]. Subjects may struggle with recall or be defensive and understate their intake, particularly if it may be viewed as excessive or problematic and/or they are not seeking treatment. This may explain why some patients reported consuming more alcohol during the structured interview than in the consultation, as they may have regarded the interview as not directly involved with their medical care. Furthermore, it has been reported that electronic administration of the AUDIT-C questionnaire was more likely to identify at-risk drinking than the same questionnaire administered in person or on paper[55]. Therefore, despite a structured interview, alcohol consumption may have been under estimated in our study. An important limitation of the study was that the number of patients who refused to participate was not recorded, which could have introduced bias, particularly as this group may have included patients more likely to not want to declare their alcohol consumption. The selection of patients also favoured English-speaking patients, introducing an ethnic and likely aetiological bias. Furthermore, bias may have been introduced as consultants were informed about the research project a year before. However, there was no difference in the number of informative alcohol histories at the initial hepatology consultation for those patients seen as new patients during the study period compared to those seen as a follow-up consultation.

Further studies could investigate the role of screening tools prior to consultation, with or without assistance, and the use of hand held computers, which may address some of the weaknesses of alcohol surveys. Although screening questionnaires are more valid and cost effective than blood screening methods[33], the low uptake in clinical practice and problems with reliability

emphasise the need for an objective biomarker to identify and evaluate drinking behaviour. An alcohol biomarker could facilitate acquiring an accurate alcohol history from patients with CLD, providing reliable evidence to challenge the patient, and could be used for monitoring and encouraging patients as they reduce their alcohol consumption.

Considerable work has been invested in identifying objective biomarkers of alcohol use. The most specific serum biomarker for heavy alcohol consumption is CDT, which, with the introduction of a standardised HPLC method, has increased clinical utility. However, its applicability to patients with liver disease was unclear, particularly since di-tri bridging was reported in patients with cirrhosis[132,133], which could result in falsely high results due to inaccurate quantification. To determine whether alcohol-independent factors influence %CDT in patients with CLD a retrospective study using serum matched to liver biopsies was performed (Chapter 4). In contrast to previous reports[132,133] there was no evidence of di-tri bridging in our cohort that would influence the interpretation of CDT results. There was also no significant affect on %CDT results by stage or aetiology of non-alcoholic disease. %CDT positively correlated with alcohol consumption, but there was a broad distribution of %CDT in non-drinkers, confirming its limited use for identifying heavy alcohol consumption. Like many studies investigating CDT, our initial CDT study had few patients consuming amounts of alcohol expected to cause a %CDT>1.7. However, in this group of patients we observed that BMI significantly influenced CDT, with elevated BMI being associated with a non-diagnostic CDT result, despite heavy drinking. Similar findings had previously been reported with older methods of CDT measurement[123-125], but also more recently with the standardised HPLC technique[118]. Although this latter study concluded that the differences were minor and not clinically relevant[118]. In our study the relationship between serum %CDT and self-reported history of alcohol consumption was examined, however alcohol histories were corroborated by a longitudinal review of the alcohol history in the medical record, blood tests and the liver biopsy findings. Patients with a CDT >1.7 who did not acknowledge heavy alcohol consumption were confidently identified to be drinking heavier than reported, ensuring that 5 patients were not regarded as having false

positive CDT results. However, the inclusion of their results would have affected the analysis, particularly with regards to the influence of alcohol consumption and volume of distribution of alcohol on %CDT.

To further investigate the influence of BMI on CDT, a prospective study (Chapter 5) involving patients who reported sustained heavy alcohol intake was undertaken. This study confirmed the initial CDT study findings regarding BMI, and demonstrated that the negative affect of BMI on %CDT was independent of other clinical variables. Other factors that independently explained the poor sensitivity of %CDT in this cohort were female gender and the presence of cirrhosis. Although similar observations have previously been described [114,134,479,481,487,547], opposing results have also been reported[132,133,486]. Therefore, despite the new standardised technique using HPLC, caution should be applied when ordering and interpreting this assay in patients with CLD, due to the poor sensitivity. Indeed, based on our findings the role of CDT is limited in the assessment of alcohol consumption in patients with liver disease since it is not reliable in the assessment of patients with liver cirrhosis being considered for liver transplant, monitoring alcohol abstinence in patients with cirrhosis, determining the role of alcohol in obese patients with fatty liver disease or as a cofactor in patients with liver disease. Its lack of sensitivity also raises concern with regards to its role in forensic analysis, e.g. the return of driving licences to those caught drink driving.

Further studies are hence needed to assess the sensitivity of %CDT with larger numbers of well-characterised patients, who consume amounts of alcohol expected to cause %CDT>1.7, a significant weakness of prior investigations. Given the utility of %CDT is limited to heavy alcohol consumption, further work could also investigate and update the mathematical equation incorporating γ-GT and %CDT[137-139] (following development of the standardised technique), to improve sensitivity and potentially identify patients consuming lower levels of alcohol that may still be harmful.

Development and validation of non-invasive biomarkers to detect, quantify and monitor hepatic fibrosis is important in the management of patients with CLD, to ensure that liver biopsy is reserved to the few cases it is needed to aid diagnosis (e.g. hepatic iron index, differentiating the cause for deranged liver

function tests in mixed disease). Identification of advanced liver fibrosis is paramount as it is associated with the majority of morbidity and mortality related to CLD and would enable management to be optimised and screening procedures implemented. In Australia, the most common non-invasive test currently employed is TE as it offers a simple, safe and efficient way to estimate hepatic fibrosis[239], but its availability is often limited to hepatology centres and its accuracy is affected by a number of factors[247,548]. Complex serum tests are minimally invasive and are now more readily available, but are still not frequently used, likely due to cost and lack of confidence in accuracy. The ELF test has recently become commercially available in Australia, despite no previous validation in an Australian cohort.

The ELF study presented in Chapter 6 is the first to assess the diagnostic accuracy of the ELF test in a large cohort of Australian patients. In patients with CLD of mixed aetiology an ELF score ≥9.8 (manufacturer's cut-off) correctly identified 74.4% of patients with advanced fibrosis and correctly excluded 92.4% of patients without advanced fibrosis, in keeping with other international studies (Table 9.1). As previously reported[201], inflammation and age negatively influenced the performance of ELF score, however our study also demonstrated the better performance of the ELF test in the presence of steatosis, not previously described. This is a significant finding in view of the global NAFLD epidemic, since many patients can remain undiagnosed until development of complications e.g. HCC. Interestingly the presence of coarse SSF, a histopathological signature of NASH, was significantly associated with metabolic complications, but did not further explain ELF scores that were discordant to the fibrosis stage.

Table 9.1. Diagnostic performance of ELF test in different aetiologies of liver disease

Study	Aetiology	Patients (n)	Fibrosis Stage	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUROC (95% CI)
Guha, I. *et al* [197]	NAFLD	192	≥ F3 (METAVIR)	0.3576†	80	90	71	94	0.90 (0.84-0.96)
Friedrich-Rust, M.et al [193]	HCV, HBV, PBC	74	≥ F3 (METAVIR)	10.22	74	70	64	79	0.79 (0.67-0.91)
Parkes, J. *et al* [199]	HCV	347	F4-6 (Ishak) ≥ F3 (METAVIR)	10.48	62	89	73	83	0.85 (0.80-0.89)
Kim, B. *et al* [202]	HBV	170	≥ F3 (METAVIR)	9.40	84	78	79	82.5	0.86 (0.81-0.92)
Wahl, K. *et al* [219]	Mixed	102	F5-6 (Ishak)	9.40	100	77	NR	NR	0.93 (0.88-0.99)
Guechot, J. *et al* [200]	HCV	512	≥ F3 (METAVIR)	9.33	90	63	73	85	0.82 (0.78-0.86)
Lichtinghagen, R. *et al* [201]	HCV	79	≥ F3 (Ishak)	9.80	85	75	NR	NR	0.90 (0.86-0.94)
Wong, G. *et al* [260]	HBV	238	≥ F3 (METAVIR)	9.80	62	66	55	72	0.69 (0.63-0.75)
Trembling, P. *et al* [496]	HBV	182	≥ F3 (METAVIR)	9.88	60	83	67	78	0.80 (0.73-0.87)
Fagan, K *et al* [549]	Mixed	329	≥ F3 (METAVIR)	9.80	74	92	75	92	0.91 (0.88-0.95)

Table 9.1 notes. †Diagnostic threshold. *Abbreviations: PPV, positive predictive value; NPV, negative predictive value; AUROC, area under the receiver operating curve; NAFLD, non-alcoholic fatty liver disease; HCV, chronic hepatitis C; HBV, chronic hepatitis B; PBC, primary biliary cirrhosis; NR, not recorded.*

The ELF study was performed in the context of a hepatology clinic and suffered from selection bias, due to the need for liver biopsy. Consequently there was a higher prevalence of viral hepatitis and in comparison to the general community, more patients with marked fibrosis. The problems of the semi-quantitative nature of histological staging systems may have been augmented by the use of the 5 stage modified METAVIR score, and would likely have been less significant if a 7 stage modified Ishak staging system had been used. Further studies are therefore required to assess the performance and utility of ELF score in patients with NAFLD, particularly in a primary care cohort setting.

Currently there is substantial variability in the cut-off used for advanced fibrosis in studies to assess ELF score, which makes comparison of results between studies difficult. To reduce this heterogeneity between studies, our study used the manufacturer's cut-off (≥9.8) to identify advanced fibrosis. In our patient cohort (*n* = 329), values of ELF score in the range 8.9–10.0 gave close to optimal cut-off points, with 9.4 being the optimal (Youden Index = 0.68), which was almost identical (0.67) to the manufacturer's 9.8 cut-off and so there was little evidence to suggest a more suitable cut-off using our dataset. Furthermore, our data could not differentiate disease-specific cut-points and it was felt that it may not be meaningful since other comorbidity is so frequent in clinical practice. A study with larger numbers of subjects for each disease aetiology would help determine whether disease specific cut-off points are required, but clearly this is difficult if liver biopsies are needed as the gold standard.

Longitudinal clinical outcome data were not available for this current study, but are clearly important data to collect in future studies in order to interpret ELF score in clinical practice. Development and validation of algorithms to cost-effectively detect fibrosis are also needed, as although simple biomarkers are not as sensitive or specific at diagnosing advanced fibrosis than complex panels, they are good at excluding advanced fibrosis and are generally cheaper. Currently the main limitation of available biomarkers used to detect fibrosis is their poor performance when there is less advanced fibrosis, possibly due to the increased relative influence of inflammation. The application of an "inflammatory factor", derived from simple tests, to negate the influence of

inflammation on the ELF score could be assessed. Novel discovery approaches, based on multiplex screening technologies may also help discover parameters that could be used to provide sensitive and specific biomarkers of fibrosis, inflammation and related pathological processes. This approach may also provide insight into the pathophysiological mechanisms driving progressive fibrosis, and could provide targets for therapeutic strategies.

The development of cirrhosis, particularly decompensated cirrhosis with ascites, is associated with a significant increase in morbidity and mortality, hospital admissions and health care costs[274]. Information regarding factors that predict hospital readmission may allow better coordination of care and patient outcomes. In a retrospective study (Chapter 7) we confirmed that medical care for decompensated cirrhosis and ascites is complex, and patients with CLD often have comorbidities that increase the burden of illness and use of healthcare resources[509,510]. The study identified that although patients with cirrhosis and ascites comprise only a relatively small population they account for a substantial use of hospital services. Markers of liver disease severity identified patients at increased risk of early readmission. The study corroborated previous findings that MELD can predict early readmission following hospital discharge for patients with decompensated cirrhosis[388,397], likely reflecting the fact that MELD indicates deterioration in both liver and renal function. Importantly the study supported that better identification of patients with poor outcomes could improve coordination of patient care, resulting in better patient outcomes and cost savings.

Although the study was limited to one calendar year at one hospital, and thus a small cohort of patients, it provided valuable information regarding the local population. The findings support the need for a change in management for these patients and in view of current literature[388] efforts should be directed at multicentre studies to assess the impact of implementing chronic disease management plans for patients with cirrhosis and ascites, similar to models used in congestive heart failure and chronic obstructive pulmonary disease[505,506]. However, although MELD may predict early readmission for patients with decompensated cirrhosis, more sensitive and specific biomarkers

are needed to predict clinical outcomes in this population. Bacterial DNA has previously been shown to be an indicator of poor prognosis in patients with cirrhosis[441], but this finding was not confirmed in a subsequent study[432]. A prospective study was thus designed to investigate the role of bacterial DNA in identifying patients with poor clinical outcomes and to establish the relationship between innate immune function, amount of bacterial DNA, microbial community composition and clinical outcomes (Chapter 8).

In keeping with the findings of Zapater *et al.*[441] the study demonstrated that levels of ascites bacterial DNA were significantly positively correlated with poor clinical outcomes and may thus be a useful biomarker. There was a negative correlation between microbial burden and ascites total protein, but not with serum markers of renal impairment, liver failure, or markers of infection/inflammation (CRP, PCT). This may suggest that the microbial burden reflects consequences of portal hypertension rather than active infection. Microbial burden also correlated positively with ascites neutrophils, which could reflect the lack of sensitivity of the cut-off of ≥250/mm^3, subclinical infection or perhaps suggest impaired neutrophil activity[356] or the presence of Gram positive infection, which are reported to have lower, non-diagnostic ascitic neutrophil counts[436]. Interestingly, consistent with the hypothesis that innate immune function is impaired in patients with cirrhosis, HLA-DR expression on ascites monocytes/macrophages inversely correlated with bacterial DNA levels. Assessment of bacterial communities found a broad phylogenetic range, similar to other studies, and that it reflected the intestinal dysbiosis previously described in patients with cirrhosis[335,336,338].

Future studies will require well characterised large patient cohorts, ideally multicentre to prevent population and laboratory bias. The studies should focus on further understanding the interplay between the innate immune system and the bacterial community and their influence on clinical outcomes. Better understanding of this may provide therapeutic strategies, which could include the use of adjunct immunomodulatory therapy to improve innate immune function. Longitudinal assessment of patients with repeated paracenteses could also be performed to monitor the change in microbial burden, bacterial

community and innate immune function with time and therapeutic interventions. One of the main difficulties with the current method is the low proportion of bacterial DNA to human DNA in the ascites specimens, which could be addressed by methods to enrich for bacterial DNA[550]. This may allow deeper assessment of the microbial diversity. Future studies could also extract bacterial DNA from serum, to compare bacterial density and diversity to that in ascites, and to further assess the application of bacterial DNA in predicting outcomes of patients with cirrhosis, particularly in the diagnosis of minimal hepatic encephalopathy.

Overall the work presented in this thesis has confirmed the need for development and validation of biomarkers for management of patients with CLD. The studies have emphasised the importance of alcohol assessment in patients with CLD and the substantial morbidity and mortality that patients with CLD incur. The studies have exhibited a number of common problems encountered in the assessment of biomarkers (e.g. imperfect gold standard, introduction of bias due to study design) and highlighted the importance of validating biomarkers in specific cohorts. They have contributed to determining the clinical utility of %CDT and the ELF test and demonstrated the potential role of bacterial DNA in the management of patients with CLD. Finally, the work has identified areas for further investigation that may eventually lead to better clinical outcomes for patients with CLD.

APPENDICES

APPENDIX 1

PATIENT INFORMATION AND CONSENT FORM

Metro South Health

Ipswich Road
Woolloongabba
Queensland 4102
AUSTRALIA

Enquiries to: Prof E. Powell PhD, FRACP
Telephone: 07 3443 8015
Facsimile: 07 3176 1295
Clinical Research Coordinator: Leigh Horsfall
07 3443 7929

THE CENTRE FOR LIVER DISEASE RESEARCH
Department of Gastroenterology & Hepatology, PAH
and
School of Medicine, UQ

Patient Information and Consent Form

Project title: Factors affecting the progression of liver disease and response to treatment.

You are invited to participate in a study investigating whether different genes or proteins in your body cells can affect the outcome of liver disease and your response to treatment. The nature of the study, requirements and other information is discussed below. Please feel free to ask any questions you may have of those discussing this research project with you.

Information: Chronic liver disease is a major health problem in Australia. Many different conditions can cause liver damage, including viral infections, alcohol, excess body fat and inherited diseases such as iron overload. Although these conditions initially affect the liver in different ways they can all produce liver fibrosis (excess scar tissue), which can eventually result in cirrhosis and liver failure. Compared with the treatment of other diseases, there are relatively few therapies available for chronic liver disease. In some liver diseases like fatty liver disease and viral hepatitis, additional therapies are urgently required.

The Centre For Liver Disease Research (CLDR), a collaborative group involving the Department of Gastroenterology and Hepatology at the Princess Alexandra Hospital and the School of Medicine at The University of Queensland, is studying a number of factors that may affect the progression of liver diseases and their response to treatment. We invite all patients who have suspected or diagnosed liver disease to participate in these studies, as the information gained from them may help us find new ways of treating, diagnosing or assessing the severity of chronic liver diseases. Any research using your samples would be with the approval of the Metro South Hospital and Health Service, Human Research Ethics Committee. Any commercial product developed as a result of this research will remain the property of the sponsor/investigator.

Queensland Government

Requirements: A small sample of blood (up to 30mls) is requested before you commence treatment of your liver disease, and possibly on one or two occasions during treatment.

If you have a liver biopsy as part of your investigation or management, a small fragment (<5mm) will be stored for research. If you have previously had a liver biopsy, we request your permission to obtain a small section from your liver biopsy that is stored in the Pathology Department.

If you undergo any liver surgery (including a liver transplant), we request that a small portion of the tissue removed during your surgical procedure is donated for research. The tissue removed during a surgical procedure (operation) on the liver will be sent to the Pathology Department for routine tests. The results of these tests will be given to your doctor and will be used to plan your post-operative care. It is usual that not all of the liver tissue removed at the time of your procedure is required for your diagnosis. We would like to collect and store small portions (approximately 100 grams) of fresh, frozen or fixed tissue, already removed during your diagnostic or surgical procedure, for the purpose of isolation of liver cells and for future molecular and genetic research. This tissue is collected in such a way that it will not interfere with your surgery, subsequent treatment or the pathology department's examination of the specimen.

Other specimens that may be collected and used to investigate the effects and/ or progression of liver disease include; urine, faeces, saliva, mouth cheek swab and / or ascites (if ascitic tap is performed for clinical indications). If you are having venesections performed as treatment for your liver disease, we request your permission to use the blood that would be otherwise discarded. These samples may provide some insight into the effect of liver disease on other parts of the body, and thus be possible non-invasive identifiers of liver disease and its severity. If we ask you to provide one or more of these samples, instruction in regards to collection will be given by the study nurse.

We may ask you to fill in a brief survey form (this would take around 30 minutes) and we may need to access your medical records and pathology laboratory results to check the details of your liver disease and treatment.

These samples (and liver tissue if available) will be used to investigate what proteins your liver produces and whether you have inherited any genes that may affect the way your body responds to your liver disease. Some of the samples may be used to investigate whether a change in the expression of factors or cells in other tissues can be used to help make a diagnosis of liver disease or determine the severity of liver disease. The specimens for genetic research will be de-identified and will be stored for as long as possible. The results of these tests will be made available to you if you request it, but will not change the way you are managed in the Clinic or influence whether you receive a liver biopsy, liver surgery, or are prescribed any medications. Any future gene studies would be approved by the relevant Human Research Ethics Committee.

Contacts

Patients who wish to enter the study or those who have already consented, can contact the Principal Investigator, Dr Elizabeth Powell at any time during the study period on 07 3443 8015 or by email at Elizabeth_Powell@health.qld.gov.au. This study has been reviewed and approved by the Metro South Hospital and Health Service ,Human Research Ethics Committee. Should you wish to discuss with someone not directly involved, in particular in relation to matters concerning policies, information about the conduct of the study or your rights as a participant, or should you wish to make an independent complaint, you may contact the Human Research Ethics Committee Coordinator, Metro South Hospital and Health Service Qld 4102 on Telephone (07) 3443 8049. Or alternatively the Princess Alexandra Hospital Patient Liasion Officer – 07 3176 5598
Email: PAH_PLO@health.qld.gov.au

This study adheres to the Guidelines of the ethical review process of The University of Queensland. Whilst you are free to discuss your participation in this study with project staff (contactable on 3176 2035), if you would like to speak to an officer of the University not involved in the study, you may contact the Ethics Officer on 3365 3924.

Version 4.1, 9August2013

PARTICIPANT CONSENT FORM

Project Title: Factors affecting the progression of liver disease and response to treatment.
Informed Consent:

To proceed with the above study, you must have read the above patient information sheet and also read and understand the following principles:

1. I confirm that I have read and understand the information sheet dated 9August2013(Version 4.1)for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.If I do withdraw my consent, I reserve the right to have my blood and tissue samples destroyed.

3. I understand and give permission for my medical records and pathology results to be reviewed in relation to this research study, in order to check details of my liver condition and treatment.

4. I agree to donate the following samples to the Centre for Liver Disease Research

   - Blood (up to 30ml) in conjunction with a routine blood test, at the time of liver biopsy / surgery and possibly on other occasions
   - Liver tissue
   - Faeces
   - Urine
   - Saliva
   - Mouth cheek swab
   - Ascitic fluid
   - Blood removed at therapeutic venesection

The samples that I donate as part of the study may be used to discover new methods of diagnosing or treating liver disease. These methods may have some commercial potential in the future. I donate my tissue freely for these purposes and waive any claim by myself and my descendants to commercial rights arising from this work.

5. I understand that genetic analysis may be carried out on DNA from my samples and that any genetic testing is related only to liver disease.

6. I agree that my samples can be stored and used in future related research.

7. I agree that my samples can be stored and used in future unspecified research.

8. I agree that my de-identified data, blood and tissue samples may be shared with other national and international liver research groups.

9. Information storage will be totally confidential and the privacy of the individual will be protected at all times. The information will be retained for at least 20 years or until there is no longer any interest.

If you fully understand all of the above information and would like to enter the study, please sign the consent below.

Name of Patient	Date	Signature

Name of Researcher	Date	Signature

Patient Sticker

APPENDIX 2

STRUCTURED ALCOHOL QUESTIONNAIRE AND DATA COLLECTION TOOL

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Alcohol RESEARCH

Today's Date: ...../...../.....

Thank you for agreeing to help with this research project. The information that you provide will remain confidential and help advance our knowledge.

We would like to know your recent daily alcohol intake. We know that this can be difficult. If you cannot recall exactly, please give it your best guess. We will help you to fill in the Calendar below and then ask you some general questions. **Please then have the attached blood test in the department today.**

To use the Alcohol Calendar: Find today's date on the calendar. Write today's day in the corresponding grey box at the top. Complete the days of the week along the top. Work **backwards** (to the left and up) from today and write in each box the number of standard drinks you had each day. Use the laminated card provided to help work out the standard drink. Fill every box until you have completed **5 rows**. If you had no alcohol at all on a day, please record "0".

Day of week	Day of week	Day of week	Day of week	Day of week	Day of week	Day of week
				1	2	3
4	5	6	7	8	9	10
11	12	13	14	15	16	17
18	19	20	21	22	23	24
25	26	27	28	29	30	31

(Jan 31, Feb 29, March 31, Apr 30, May 31, Jun 30, July 31, Aug 31, Sept 30, Oct 31, Nov 30, Dec 31)

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Alcohol RESEARCH

Today's Date: ...../...../.....

What type of alcohol do you usually drink? _____

Does the calendar reflect your usual alcohol consumption? **Y / More / Less**

(Specify, particularly month before calendar) _____

_____

How many grams / week of alcohol do you currently drink? [ ]

Have you significantly reduced your alcohol recently? **Y / N** (specify)

_____

_____

Have you ever drunk alcohol heavily? (specify) _____

_____

_____

Caffeine per day (mg)? [ ] (specify) _____

_____

Smoker? Never / Ex / Yes No. / day [ ] Years [ ] Pack years [ ]

Recreational drugs: IVDU? Never / Ex / Yes (specify) _____

MJ: Never / Ex / Yes (specify) _____

Other: _____ _____

_____

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Alcohol RESEARCH

Today's Date: ...../...../.....

CDT Prospective

Chart review and patient interview

Height ☐ Weight ☐ Waist Circumference ☐

BMI ☐ Age ☐ Gender ☐

Ethnicity: Caucasian / Asian / ATSI / African / Other (specify) _____

Actual Liver Diagnosis: _____

Hyperlipidaemia ☐ Hypertension ☐ IHD ☐

Psoriasis ☐ Asthma ☐ Rheumatoid ☐

Transplant **Y / N** (specify) _____ Malignancy **Y / N** (specify) _____

Thyroid disease **Y / N** (specify) _____ Diabetes Mellitus **Y / N** (specify) _____

Other medical problems: _____

_____

_____

_____

Medications: _____

_____

_____

_____

OTC/herbal Meds: _____

_____

UR..... ☐ Female ☐ Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Alcohol RESEARCH

Today's Date: ...../...../.....

1) At what age did you start drinking alcohol on a regular basis?

2) Please specify below any periods of your life when you drank alcohol heavily.

3) Please write in each box how many standard drinks you would usually have per day over a two week period.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Wk1							
Wk2							

If you do not drink alcohol every week, please specify below how many standard drinks you would drink per month.

AUDIT

1) How often do you have a drink containing alcohol?

Never	Monthly or less	2 to 4 times a month	2 to 3 times a week	4 or more times a week

2) How many "standard" drinks containing alcohol do you have in a typical day when you are drinking?

1 or 2	3 or 4	5 or 6	7 to 9	10 or more

3) How often do you have six or more drinks on one occasion?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily

UR..... ☐ Female ☐ Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Alcohol RESEARCH

Today's Date: ...../...../.....

4) How often during the last year have you found that you were not able to stop drinking once you started?

Never | Less than monthly | Monthly | Weekly | Daily or almost daily

5) How often during the last year have you failed to do what was normally expected from you because of drinking?

Never | Less than monthly | Monthly | Weekly | Daily or almost daily

6) How often during the last year have you needed a drink in the morning to get yourself going after a heavy drinking session?

Never | Less than monthly | Monthly | Weekly | Daily or almost daily

7) How often during the last year have you had a feeling of guilt or remorse after drinking?

Never | Less than monthly | Monthly | Weekly | Daily or almost daily

8) How often over the last year have you been unable to remember what happened the night before because you had been drinking?

Never | Less than monthly | Monthly | Weekly | Daily or almost daily

9) Have you or someone else been injured as a result of your drinking?

Never | Yes, but not in the last year | Yes, during the last year

10) Has a relative, a friend, a doctor or other health worker been concerned about your drinking or suggested you cut down?

No | Yes, but not in the last year | Yes, during the last year

UR..... ☐ Female ☐ Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Alcohol RESEARCH

Today's Date: ...../...../.....

BMAST - 6	Yes	No
• Do you feel you are a normal drinker?	☐	☐
• Do friends and relatives think you are a normal drinker?	☐	☐
• Have you ever attended a meeting of AA?	☐	☐
• Have you ever lost friends or girlfriends/boyfriends because of drinking?	☐	☐
• Have you ever been in trouble at work because of drinking?	☐	☐
• Have you ever neglected your obligations, your family, or your work for two or more days in a row because of drinking?	☐	☐
• Have you ever had "DTs", severe shakes, heard voices or hallucinated after heavy drinking?	☐	☐
• Have you ever gone to anyone for help about your drinking?	☐	☐
• Have you ever been in hospital because of drinking?	☐	☐
• Have you ever been arrested for drink driving?	☐	☐

Thank you for completing this. Please give this to your doctor when you go in to the clinic room.

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Alcohol RESEARCH

Today's Date: ...../...../.....

Referral from: **GP** (postcode)/**Specialist** (specify) _____ Date: ____

Reason for referral: _____

Alcohol Hx included: **Y / N** (documented details)_____

_____

1st Hepatology Consultation Date: ____ Staff Grade: ____

Alcohol Hx included: **Y /N** (documented details) _____

_____

_____

Review of chart: (documented alcohol consumption with dates) _____

_____

_____

Previous Heavy Alcohol? **Y / N** (specify) _____

_____

Today's Consultation Date: ____ Staff Grade: ____

Alcohol History included : **Y / N** (specify)_____

_____

Smoking Hx Documented: **Y / N** _____Ever **Y/ N** (Specify)_____

APPENDIX 3

ALCOHOL DATA COLLECTION TOOL

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Alcohol RESEARCH

Biopsy Date: ...../...../.....

DiTri Bridging Study

Height ☐ Weight ☐ Waist Circumference ☐

BMI ☐ Age ☐ Gender ☐

Ethnicity: Caucasian / Asian / ATSI / African / Other (specify) _____

Liver Diagnosis: _____

Hyperlipidaemia ☐ Hypertension ☐ IHD ☐

Psoriasis ☐ Asthma ☐ Rheumatoid ☐

Transplant Y / N (specify) _____ Malignancy Y / N (specify) _____

Thyroid disease Y / N (specify) _____ Diabetes Mellitus Y / N (specify) _____

Other: _____

_____

_____

Medications: _____

_____

_____

Smoker? Never / Ex / Yes No. / day ☐ Years ☐ Pack years ☐

Recreational drugs/OTC meds: _____

_____

_____

_____

Referral from: _____ Date: [ ]

Reason for referral: _____

Alcohol Hx included: Y / N (specify) _____

_____

Hepatology Consultation Date: [ ] Grade: [ ]

Alcohol History: Y /N (specify) _____

_____

_____

Previous Heavy Alcohol? Y / N (specify) _____

_____

_____

Alcohol around Biopsy: _____

_____

_____

How many grams / week at biopsy? [ ]

Have they significantly reduced their alcohol recently? Y / N (specify)

_____

6 Months prior? _____

Liver Bx book: _____

Anaesthetic Q: _____

Other: _____

_____

2

APPENDIX 4

ELF DATA COLLECTION TOOL

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

ELF RESEARCH

Biopsy Date: ...../...../.....

Height ☐ Weight ☐ Waist Circumference ☐ Gender ☐

Ethnicity: Caucasian / Asian / ATSI / African / Other (specify where born)_____

Actual Liver Diagnosis: _____

Any Liver Cofactors: _____

At time of biopsy: AKI ☐ _____ ALF ☐ _____

Rheum Arthritis ☐ Psoriasis ☐ Chronic Pancreatitis ☐ Fibrosis (specify) ☐

After biopsy:_____

Hyperlipidaemia ☐ Hypertension ☐ OSA ☐

CKD ☐ IHD ☐ Asthma/COPD (delete) ☐

At time of biopsy: Pulse ☐ BP ☐ **Clinic & prior**: _____

Transplant **Y / N** (specify) _____ Malignancy **Y / N** (specify) _____

Thyroid disease **Y / N** (specify) _____ Diabetes Mellitus **Y / N** (specify) _____

Other: _____

_____

Metformin ☐ 6MP ☐ Methotrexate ☐ Insulin_____

Propanolol ☐ Frusemide ☐ Spironolactone ☐ PPI _____

Azathioprine ☐ TNFα inhib _____ Thiazolidinedione _____

Clopidogrel ☐ Fish oil ☐ Aspirin ☐ Warfarin ☐ Steroids_____

Duration steroids/CFAW stopped before bx_____

Medications/OTC meds: _____

_____

_____

Initial Hepatology Consult Date: [ ] Grade: [ ]

Alcohol History: **Y /N** (specify) _____

_____

_____

Previous Heavy Alcohol? (♀≥350g/wk, ♂≥420g/wk for >6months) **Y / N** (specify)

_____

_____

Alcohol around Biopsy: _____

_____

_____ **How many grams / week at biopsy?** [ ]

Have they significantly reduced their alcohol recently (last month)? **Y / N** (specify)

_____

6 Months prior? _____

Liver Bx book: _____

Anaesthetic Q: _____

Smoker? Never / Ex / Yes No. / day [ ] Years [ ] Pack years [ ]

Recreational drugs: IVDU? Never / Ex / Yes (specify) _____

MJ: Never / Ex / Yes (specify)_____ Other:_____

Other: _____

_____

_____

2

*Most recent blood tests around **BIOPSY***

U&Es Date: **LFTs** Date: **FBC** Date: **Coag** Date:

Na		Alb		Hb		INR	
K		TBili		Conj		Plt		PT	
Gluc		ALP		MCV		APTT	
Urea		GGT		WCC		**HFE** Date:	
Creat		ALT		Neut		& result	
eGFR		AST					

Other: HDV:

Liver screen – closest result to biopsy

Date:	T4:	TSH:	Date:	Ceruloplasmin:	umol/l	Date:	AFP:		
Date:	A1AT:	Phenotype:	Date:	TFN:	TFN Sats:	Ferritin:			
HepB Date:	DNA:	x10	**eAg**:neg/pos	**sAg**:neg/pos	sAb:	cAb:			
HCV Date:	IgG: neg/pos	Date:	RNA:	x10	Genotype:	IL28B:			
Date:	ANA:	SMA:	AMA:	LKM:	Date:	HIV:neg/pos			

Ultrasound Date: Hepatomegally **Y / N** (size)_____ Retrograde flow **Y / N**
Varices **Y / N** Splenomegally **Y / N** (size)_____

Other Imaging: _____

_____

Fibroscan Date: Liver Stiffness: kPa CAP

% Valid readings: Probe Size: IQR: Waist Circumference:

OGD Date: Varices **Y / N** Size: PHT Gastropathy **Y / N**

Other: _____

3

Estimated duration of disease: _____

HCV: _____ Yrs

HBV: _____ Yrs

Cirrhosis: _____

Development of HCC? **Yes / No** Estimated date of development:

If No HCC, document last date seen in hepatology clinic:

Other Comments

Outcomes: What treatments have they had?_____

APPENDIX 5

ASCITES DATA COLLECTION TOOL

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Ascites Audit

Date of Ascitic Tap: ...../...../.....

Retrospective / Prospective

Height ☐ Weight ☐ Waist Circumference ☐

BMI ☐ Liver Diagnosis_____

Ethnicity: *Caucasian / Asian / ATSI / African / Other* (specify) _____

Albumin ☐ Bilirubin ☐ INR ☐ Ascites ☐ HE ☐

Child Pugh Score ☐

Creatinine ☐ Bilirubin ☐ INR ☐ Dialysis twice per week **Y / N**

MELD Score ☐

Date of Hospital Admission _____ Date of Discharge/Death _____

Length of Hospital Admission (days) ☐

Reason for Hospital Admission: *Ascites / Other* (specify) _____

Reason for tap: ☐ ? SBP (patient unwell/sepsis) ☐ Diagnostic (? SAAG/cytology)

☐ Post Abx for SBP ☐ Therapeutic ☐ Other (specify) _____

Patient symptoms: *Fever / Abdominal Pain / Confusion / Sepsis / Other*(specify)

_____

Date 1st ascitic tap _____ No. taps last 12mths ☐ (08/10/11 – 12)

Total no. taps performed ☐ Prev SBP **Y / N** On prophylactic abx **Y / N**

Tap performed: *Gastro ward / other ward / ultrasound* By (Staff Grade) ☐

U/S guided / Marked up / Blind Most recent ultrasound prior ☐

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Ascites Audit

Date of Ascitic Tap: ...../...../.....

Retrospective / Prospective

Complications: Intraprocedure **Y / N** (specify) _____

Post procedure **Y / N** (specify) _____

Amount of fluid drained? (ml) ☐ % albumin used ☐ Amt infused (ml) ☐

If SBP: Antibiotics used _____ Course (days) ☐

Started on prophylactic abx **Y / N** ☐

CKD ☐ eGFR on day ☐ Hypertension ☐ IHD ☐

Diabetes Mellitus **Y / N** (specify) _____ Malignancy **Y / N** (specify) _____

Transplant **Y / N** (specify) _____ Prev. Bowel Surgery **Y / N** (Specify) _____

Other medical/surgical history: _____

_____

_____

PPI /Antacid **Y / N** (specify) _____ Laxatives **Y / N** (specify) _____

Propanolol **Y / N** (specify) _____ ACEI **Y / N** (specify) _____

Antibiotics within 2/12 (specify duration, last dose and which) _____

_____

Anticoagulant **Y / N** (specify) _____ Aspirin ☐ NSAIDs ☐ Fish Oil ☐

Other medications: _____

_____

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Ascites Audit

Date of Ascitic Tap: ...../...../.....

Retrospective / Prospective

Current alcohol/week (g/wk) ☐

Prev alcohol excess **Y / N** (specify) _____

Smoker? Never / Ex / Yes No. / day ☐ Years ☐ Pack years ☐

Fluid Chemistry:

Protein ☐ g/L

Glucose ☐ mmol/L

LDH ☐ U/L

Albumin ☐ g/L SAAG ☐

Body Fluid Examination:

Appearance ☐

Cell Count: WBC ☐ RBC ☐

Viscosity (%) ☐

Polymorphs ☐ Eosinophils ☐

Mononuclears ☐ Others ☐

Gram Stain ☐

Antimic's detected ☐

Culture ☐

Comment: _____

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

Ascites Audit

Date of Ascitic Tap: ...../...../.....

Retrospective / Prospective

Any Other Relevant Information:

APPENDIX 6

ASCITES QUESTIONNAIRE AND DATA COLLECTION TOOLS

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

BT Ascites Research

Today's Date: ...../...../.....

Height ☐ Weight ☐ Pulse ☐ BP ☐

BMI ☐ Age ☐ Gender ☐ Temp ☐

Ethnicity: Caucasian / Asian / ATSI / African / Other (specify) _____

Actual Liver Diagnosis: _____

Reason for tap: ☐ ? SBP (patient unwell/sepsis) ☐ Diagnostic (? SAAG/cytology)

☐ Post Abx for SBP ☐ Therapeutic ☐ Other (specify) _____

Prev ascitic tap **Y / N** (number)_____ Prev SBP **Y / N** On prophylactic abx **Y / N**

Tap performed: *Gastro ward / other ward / ultrasound* By (Staff Grade) ☐

U/S guided / Marked up / Blind Most recent ultrasound prior ☐

Hyperlipidaemia ☐ Hypertension ☐ IHD ☐

Psoriasis ☐ Asthma ☐ Rheumatoid ☐

Transplant **Y / N** (specify)_____ Malignancy **Y / N** (specify)_____

CKD (eGFR) **Y / N** (specify)_____ Diabetes Mellitus **Y / N** (specify)_____

Previous Bowel Surgery **Y / N** (Specify)_____ Cholecystectomy **Y / N**

Other medical/surgical history: _____

_____

Previous: Variceal Bleed **Y / N** (specify)_____ Encephalopathy **Y / N** (specify)_____

PPI /Antacid **Y / N** (specify)_____ Laxatives **Y / N** (specify)_____

Propanolol **Y / N** (specify)_____ ACEI **Y / N** (specify)_____

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

BT Ascites Research

Today's Date: ...../...../.....

Antibiotics (specify duration, last dose and which) _____

_____

_____

Anticoagulant **Y / N** (specify) _____ Aspirin ☐ NSAIDs ☐ Fish Oil ☐

Steroids **Y / N** (specify duration and dose) _____

Immunosuppressant's **Y / N** (specify) _____

OTC/herbal Meds/Vitamins: _____

Other medications: _____

_____

Probiotic drinks/yoghurts (specify type and frequency of use) _____

_____

Smoker? Never / Ex / Yes No. / day ☐ Years ☐ Pack years ☐

Caffeine per day (mg)? ☐ (specify) _____

Other: _____

_____

EAT score: ☐ Comments: _____

Current alcohol/week (g/wk) ☐ Prev alcohol excess **Y / N** (specify) _____

_____

Type of alcohol usually drinks? _____ AUDIT ☐ BMAST ☐

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

BT Ascites Research

Today's Date: ...../...../.....

Eating Assessment Table – EAT 2008

Questions 1-4 describe a food group or cooking method (left column). To the right of this description are 5 different eating or cooking patterns that could describe your typical eating or cooking habits. For each of the 4 following categories on the left (Fruits, Vegetables, etc.), **circle the one** description that best describes your eating habits.

	Category						Total (For Staff Only)
1	How much **FRUIT** do you eat? A portion of fruit is about 3/4 of a cup or roughly the size of a tennis ball. Whole fruit juices count as a portion for every 4 ozs up to 2 portions per day. Dried fruit should be estimated based upon the pre-dried size of the food.	0-2 per WEEK	3-7 per WEEK	1-2 per DAY	>3 per DAY	I eat 3 or more per day. My fruit consumption is varied and includes berries, citrus, and other fruits.	
2	How much **VEG** do you eat? This category include leafy green vegetables as well as tomatoes, peppers, cucumbers, squash, eggplant, string beans, and root plants such as carrots, parsnips, and potatoes. A portion size is roughly 1 cup of raw vegetables or 1/2 cup of cooked vegetables. For the purpose of this exercise do not count potatoes in your total.	<1 per DAY	1-2 per DAY	2-4 per DAY	>4 per DAY	I eat 4 or more vegetables per day. My vegetable consumption is varied and includes leafy green vegetables as well as other vegetables. When possible I try to eat fresh or frozen vegetables rather than canned.	
3	Do you eat **LEGUMES**? This family of foods is composed of lentils and a wide variety of beans (eg kidney bean, lima beans, black beans, chickpeas, "split peas", etc.). Soy beans and tofu (which is derived from soybeans) fit in this category. A serving portion is 3/4 - 1 cup.	Rarely <1 portion per WEEK	1-2 per WEEK	2-4 per WEEK	>4 per WEEK	≥1 per DAY	
4	**COOKING METHODS** How food is prepared influences greatly its nutritional quality, mostly because the quantity and quality of fat becomes hidden in the recipe.	My food is prepared using mostly butter, shortening or vegetable oils (not olive oil or canola), and eat deep fried or breaded foods ≥ 1 per week. Or I do not know how my food is prepared.	My food is prepared using mostly butter, shortening or vegetable oils (not olive oil or canola), but my food is not deep-fried.	My food is prepared with butter shortening or other oils and sometimes with oils that are high in monounsaturated fats (eg olive oil or canola).	My food is prepared with moderate to liberal amounts of oils that are high in monounsaturated fats (eg olive oil or canola).	My food is prepared with small amounts of oils that are high in monounsaturated fats (eg olive oil or canola) or with no oil (steamed, boiled or baked, or roasted)	

Questions 5-11 pertain to nutrition knowledge or specific dietary habits. For each category on the left, (Knowledge of Fats, etc) read the phrases on the right and **please check (√) each box (□)** that applies to you.

5	**Meat** Meats can be lumped into groups: **Low Fat** Skinless chicken Lean or extra-lean cuts of beef, pork, or lamb Wild meat: moose, caribou, etc. **High Fat** Bacon Sausage Red meat unless labelled lean or extra-lean Chicken with skin or breading	The meat I eat is mostly: Low fat □ Mixed □ High fat □ I generally eat less than 6 oz of meat per wk □	I eat meat: 0-4 times/week □ 1-2 times/day □ more then 2 times/d. □	My average portion is: 0-3 oz □ 4-6 oz □ 7-9 oz □ ≥9 oz □ 3 oz = 1 deck of playing cards	

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6	**Starchy Foods** Starch is a type of carbohydrate found in plants. Common sources would be: **Grains** (cereals) rice, oats, barley, baked goods: breads, pasta **Legumes** beans, lentils **Some vegetables** Corn, Potatoes, Sweet peas 1 portion = 1 slice of bread or 1/2 cup of grains, legumes or vegetables.	**Whole grain vs Processed** When I eat grain based foods I eat: Mostly foods from whole grains ☐ A mix of whole and processed grains ☐ Mostly foods from processed grains ☐	**Recognition of starchy foods** I am able to identify the high starch foods in my pantry ☐ I am able to identify the high starch foods at a grocery store ☐ I am able to recognize high starch foods at a restaurant ☐	**Portion control** On average how many portions of starchy foods do you eat per meal?: ≤ 2 portion/meal ☐ 2-3 portions/meal ☐ 3-4 portions/meal ☐ > 4 portions/meal ☐ I don't know ☐	
7	**Dairy and replacements** Low Fat "dairy" products — portion size 0-1 % milk — 250 ml (1cup) 0-1% yogurt — 175 g (3/4 cup) Soy or rice milk — 175 ml (3/4 cup) High fat "dairy" products — portion size cheddar cheese — 50 g (1 oz = 30g) 2-3.25% milk — 250 ml (1 cup) ice cream — 3/4 cup	**Total Dairy** I eat: 0-1 ☐ 1-2 ☐ 2-3 ☐ 3-4 ☐ portions per day of dairy products	**High Fat Dairy** I eat: 0-1 ☐ 1-2 ☐ 2-3 ☐ >3 ☐ portions per day of **high fat** dairy products (butter, cream, ice cream, 2-3.5% milk or products derived from 2-3.5% milk)	**Low Fat Dairy** I eat low fat dairy products and I eat mostly products that are: Mostly 0% fat ☐ Mix of 0-1% fat ☐ Mostly 1% fat ☐	
8	**Knowledge of Fats** There are many different types of fat. Nutritional labels provide much information about fat content. This section tests your ability to read and understand this information.	I understand how to read nutritional labels looking for information about fat content Yes ☐ No ☐ I understand that trans fats may be a risk factor for heart and other diseases. Yes ☐ No ☐	I understand that in moderate amounts monounsaturated and polyunsaturated fats are "good for the heart" and that saturated fats are "bad for the heart" Yes ☐ No ☐ I am able to calculate from a nutritional label the % of calories from fat Yes ☐ No ☐	I regularly read the nutritional labels of foods that I eat to determine the quality and quantity of fat Yes ☐ No ☐	
9	**Alcohol & Omega - 3 fatty acids**	**Alcohol** How many drinks per week do you average? **Women** 0-2 per wk ☐ 3-5 per wk ☐ 6-9 per wk ☐ greater than 9 per wk ☐ **Men** 0-2 per wk ☐ 3-5 per wk ☐ 6-14 per wk ☐ greater than 14 per wk ☐	I eat fish (salmon, tuna, trout, herring, and mackerel), without breading or "heavy sauces" 0-1 per month ☐ 0-1 per week ☐ 1-2 per week ☐ greater than 2 per week ☐	I consume foods known to contain Omega 3 fatty acids such as flax seeds, walnuts, and oat or wheat germ: ☐ 0-1 times per week ☐ 1-2 times per week ☐ 2-3 times per week ☐ 3-4 times per week ☐ > 4 times per week	
10	**Other**	**Diversity** I make an effort to eat a wide variety of foods over the course of a week, and generally avoid eating excess amounts of any one food. Yes ☐ No ☐ **Portion Control** I tend to take small to medium portions of food, and rarely go back for a second helping. Yes ☐ No ☐	**Nuts** I consume small portions of nuts 3 or more times per week. Yes ☐ No ☐ **Vitamins** I take a multi-vitamin daily. Yes ☐ No ☐	**Grazing** I tend to eat multiple small snacks and meals (5 or more/day) rather than 3 or less large meals. Yes ☐ No ☐☐	

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6	**Starchy Foods** Starch is a type of carbohydrate found in plants. Common sources would be: **Grains** (cereals) rice, oats, barley, baked goods: breads, pasta **Legumes** beans, lentils **Some vegetables** Corn, Potatoes, Sweet peas 1 portion = 1 slice of bread or 1/2 cup of grains, legumes or vegetables.	Whole grain vs Processed When I eat grain based foods I eat: Mostly foods from whole grains ☐ A mix of whole and processed grains ☐ Mostly foods from processed grains ☐	Recognition of starchy foods I am able to identify the high starch foods in my pantry ☐ I am able to identify the high starch foods at a grocery store ☐ I am able to recognize high starch foods at a restaurant ☐	Portion control On average how many portions of starchy foods do you eat per meal?: ≤ 2 portion/meal ☐ 2-3 portions/meal ☐ 3-4 portions/meal ☐ > 4 portions/meal ☐ I don't know ☐	
7	**Dairy and replacements** Low Fat "dairy" products — portion size 0-1 % milk — 250 ml (1cup) 0-1% yogurt — 175 g (3/4 cup) Soy or rice milk — 175 ml (3/4 cup) High fat "dairy" products — portion size cheddar cheese — 50 g (1 oz = 30g) 2-3.25% milk — 250 ml (1 cup) ice cream — 3/4 cup	**Total Dairy** I eat: 0-1 ☐ 1-2 ☐ 2-3 ☐ 3-4 ☐ portions per day of dairy products	**High Fat Dairy** I eat: 0-1 ☐ 1-2 ☐ 2-3 ☐ >3 ☐ portions per day of **high fat** dairy products (butter, cream, ice cream, 2-3.5% milk or products derived from 2-3.5% milk)	**Low Fat Dairy** I eat low fat dairy products and I eat mostly products that are: Mostly 0% fat ☐ Mix of 0-1% fat ☐ Mostly 1% fat ☐	
8	**Knowledge of Fats** There are many different types of fat. Nutritional labels provide much information about fat content. This section tests your ability to read and understand this information.	I understand how to read nutritional labels looking for information about fat content Yes ☐ No ☐ I understand that trans fats may be a risk factor for heart and other diseases. Yes ☐ No ☐	I understand that in moderate amounts monounsaturated and polyunsaturated fats are "good for the heart" and that saturated fats are "bad for the heart" Yes ☐ No ☐ I am able to calculate from a nutritional label the % of calories from fat Yes ☐ No ☐	I regularly read the nutritional labels of foods that I eat to determine the quality and quantity of fat Yes ☐ No ☐	
9	**Alcohol & Omega - 3 fatty acids**	**Alcohol** How many drinks per week do you average? **Women** 0-2 per wk ☐ 3-5 per wk ☐ 6-9 per wk ☐ greater than 9 per wk ☐ **Men** 0-2 per wk ☐ 3-5 per wk ☐ 6-14 per wk ☐ greater than 14 per wk ☐	I eat fish (salmon, tuna, trout, herring, and mackerel), without breading or "heavy sauces" 0-1 per month ☐ 0-1 per week ☐ 1-2 per week ☐ greater than 2 per week ☐	I consume foods known to contain Omega 3 fatty acids such as flax seeds, walnuts, and oat or wheat germ: ☐ 0-1 times per week ☐ 1-2 times per week ☐ 2-3 times per week ☐ 3-4 times per week ☐ > 4 times per week	
10	**Other**	**Diversity** I make an effort to eat a wide variety of foods over the course of a week, and generally avoid eating excess amounts of any one food. Yes ☐ No ☐ **Portion Control** I tend to take small to medium portions of food, and rarely go back for a second helping. Yes ☐ No ☐	**Nuts** I consume small portions of nuts 3 or more times per week. Yes ☐ No ☐ **Vitamins** I take a multi-vitamin daily. Yes ☐ No ☐	**Grazing** I tend to eat multiple small snacks and meals (5 or more/day) rather than 3 or less large meals. Yes ☐ No ☐☐	

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11	**Empty calories**	On average I eat one or more of the following sugar containing beverages per day: soft drinks (not diet), sweetened fruit punch, sports drinks. Iced tea, etc. Yes ☐ No ☐	I eat a salty (high fat) snack at least 4 times per week: eg chips, french fries, popcorn, tortillas, cheese puffs, crackers. Yes ☐ No ☐	I eat a desert other than fruit or a low fat yogurt about 1 per day. Yes ☐ No ☐	I eat a desert other than fruit or a low fat yogurt about 2 times per day. (circle category to left as well if you circle here) Yes ☐ No ☐	Outside of my meals, I eat a sweet snack at least once per day. eg: candy or chocolate bar, cakes, cookies, high fat muffins, brownies. Yes ☐ No ☐	

EAT Score:

1) At what age did you start drinking alcohol on a regular basis?

2) Please specify below any periods of your life when you drank alcohol heavily.

3) Please write in each box how many standard drinks you would usually have per day over a two week period.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Wk1							
Wk2							

If you do not drink alcohol every week, please specify below how many standard drinks you would drink per month.

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AUDIT

1) How often do you have a drink containing alcohol?

Never	Monthly or less	2 to 4 times a month	2 to 3 times a week	4 or more times a week
☐	☐	☐	☐	☐

2) How many "standard" drinks containing alcohol do you have in a typical day when you are drinking?

1 or 2	3 or 4	5 or 6	7 to 9	10 or more
☐	☐	☐	☐	☐

3) How often do you have six or more drinks on one occasion?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily
☐	☐	☐	☐	☐

4) How often during the last year have you found that you were not able to stop drinking once you started?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily
☐	☐	☐	☐	☐

5) How often during the last year have you failed to do what was normally expected from you because of drinking?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily
☐	☐	☐	☐	☐

6) How often during the last year have you needed a drink in the morning to get yourself going after a heavy drinking session?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily
☐	☐	☐	☐	☐

7) How often during the last year have you had a feeling of guilt or remorse after drinking?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily
☐	☐	☐	☐	☐

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Given Names.....

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8) How often over the last year have you been unable to remember what happened the night before because you had been drinking?

Never | Less than monthly | Monthly | Weekly | Daily or almost daily

9) Have you or someone else been injured as a result of your drinking?

Never | Yes, but not in the last year | Yes, during the last year

10) Has a relative, a friend, a doctor or other health worker been concerned about your drinking or suggested you cut down?

No | Yes, but not in the last year | Yes, during the last year

BMAST - 6	Yes	No
• Do you feel you are a normal drinker?	☐	☐
• Do friends and relatives think you are a normal drinker?	☐	☐
• Have you ever attended a meeting of AA?	☐	☐
• Have you ever lost friends or girlfriends/boyfriends because of drinking?	☐	☐
• Have you ever been in trouble at work because of drinking?	☐	☐
• Have you ever neglected your obligations, your family, or your work for two or more days in a row because of drinking?	☐	☐
• Have you ever had "DTs", severe shakes, heard voices or hallucinated after heavy drinking?	☐	☐
• Have you ever gone to anyone for help about your drinking?	☐	☐
• Have you ever been in hospital because of drinking?	☐	☐
• Have you ever been arrested for drink driving?	☐	☐

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Today's Date: ...../...../.....

Most recent blood tests

U&Es Date:		**LFTs** Date:		**FBC** Date:		**Coag** Date:	
Na		Alb		Hb		INR	
K		Bili		Plt		PT	
Gluc		ALP		MCV		APTT	
Urea		GGT		WCC			
Creat		ALT		Neut			
eGFR		AST		Ferritin Date & result:			

Ultrasound Date: Hepatomegally **Y / N** Retrograde flow **Y / N**

Varices **Y / N** Splenomegally **Y / N** (size) _____

Other Imaging: _____

_____

Liver Biopsy Date: Fibrosis Stage: Steatosis Grade:

Activity: Fe:

Other: _____

Fibroscan Date: Liver Stiffness: kPa Fibrosis stage:

Other: _____

OGD Date: Varices **Y / N** Size: PHT Gastropathy **Y / N**

Other: _____

UR..... Female Male

Surname.....

Given Names.....

DOB...../...../..... PLACE PATIENT ID STICKER HERE

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Today's Date: ...../...../.....

Fluid Chemistry:

Protein ____ g/L

Glucose ____ mmol/L

LDH ____ U/L

Albumin ____ g/L SAAG ____

Body Fluid Examination:

Appearance ____

Cell Count: WBC ____ RBC ____

Viscosity (%) ____

Polymorphs ____ Eosinophils ____

Mononuclears ____ Others ____

Gram Stain ____

Antimic's detected ____

Culture ____

Comment: _____

Ascites Prospective

Previous

Estimated date cirrhosis development: =length of time

Comment:

Estimated date 1st ascites:

Comment:

Date 1st ascitic tap:

Comment: elsewhere?

No. taps prior to Q?

Prev SBP? Yes/No

Organism:

On Day:

Ward or *Day case* (delete)

Length of admission if ward:

Reason for admission:

Planned/Unplanned (delete)

Amount of fluid drained?

Nearest u/s:

Nearest liver biopsy

Nearest Fibroscan:

Nearest OGD:

Outcomes:

Death? Yes/No Date:

Transplant? Yes/No Date:

Development SBP in 6/12: Yes/No

Organism:

1st Readmission date:

Reason:

No. Admission in 6/12: Ascites:

Planned:

Unplanned:

Other:

Specify:

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